"COMPARATIVE EVALUATION OF ANTIMICROBIAL ACTION OF PHOTODYNAMIC DISINFECTION, DYES AND NANOSILVER AGAINST STREPTOCOCCUS MUTANS AND LACTOBACILLUS ACIDOPHILLUS ON CORONAL DENTIN- AN IN VITRO STUDY"

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Towards the partial fulfillment for the degree of

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BRANCH – IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

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Certificate

This is to certify that **Dr. M.SARAVANAN** post graduate student (2007 - 2010) in the Department of Conservative Dentistry and Endodontics, Tamil Nadu Government Dental College and Hospital, Chennai – 3, has done this dissertation titled"**COMPARATIVE EVALUATION OF ANTIMICROBIAL ACTION OF PHOTODYNAMIC DISINFECTION, DYES AND NANOSILVER AGAINST STREPTOCOCCUS MUTANS AND LACTOBACILLUS ACIDOPHILLUS ON CORONAL DENTIN- AN IN VITRO STUDY"** under our direct guidance and supervision in partial fulfillment of the regulations laid down by **The Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai – 32** for **M.D.S.** Conservative Dentistry and Endodontics (Branch **IV**) Degree Examination.

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CONTENTS

DECLARATION

TITLE OF THE DISSERTATION	**COMPARATIVE EVALUATION OF ANTIMICROBIAL ACTION OF PHOTODYNAMIC DISINFECTION, DYES AND NANOSILVER AGAINST STREPTOCOCCUS MUTANS AND LACTOBACILLUS ACIDOPHILLUS ON CORONAL DENTIN- AN IN VITRO STUDY**
PLACE OF THE STUDY	**Tamilnadu Government Dental College and Hospital, Chennai – 3**
DURATION OF THE COURSE	**THREE YEARS**
NAME OF THE GUIDE	**Dr. S.C.Loganathan**
HEAD OF THE DEPARTMENT	**Dr. S.C.Loganathan**

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COMPARATIVE EVALUATION OF ANTIMICROBIAL ACTION OF PHOTODYNAMIC DISINFECTION…….

MDS BRANCH IV

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INTRODUCTION

Caries is a transmissible, infectious illness in which diverse pathological factors have been identified , the presence of acidogenic bacteria such as *Streptococcus mutans* and lactobacilli,[76] [43]salivary dysfunction and dietary carbohydrates are related to caries progression[34,46].

Dental caries remains as one of the most widespread diseases of mankind, about 95% of the world population is affected in different ages of their lives and, in both developed and poor countries dental caries is still considered as a public health problem. It is a multifactorial infectious disease, related to biofilm accumulation on dental surface and frequent consumption of fermentable carbohydrates[39].

The disease has been characterized as an ecological collision in the mouth, involving infectious bacteria and the ready availability of sugars in the diet, in which the microbial population uses to produce destructive organic acids. If allowed to persist, the acidification brought about by bacteria results in permanent demineralization of the enamel. By the fermentation of dietary carbohydrates, the bacteria in

the dental biofilm produce acids that decrease the pH and increase the biofilm potential in promoting dental demineralization.

The microbial populations involved in dental caries are known to be highly complex and variable that has not yet been fully identified, although key organisms are generally recognized to be associated with disease progressions. The bacteria involved in caries initiation and early caries development, particularly are the mutans group especially Streptococcus and lactobacilli have been well documented[73]. The most heavily investigated etiologic agent of dental caries is streptococcus mutans, a gram-positive coccus, and also other acid-tolerant oral streptococci, lactobacillus species and also in some cases include strains of actinomycetes, involved in human dental caries and have been shown to cause caries in animal .

The focus on S.mutans is because of its consistently high correlation with disease in epidemiological surveys of children and adults, its low abundance in the absence of disease, its demonstrated ability to grow at low pH values in vitro, and its ability to cause caries in animal models. There is also an increasing evidence of an association between lactobacilli and caries in humans.

Lactobacillus represents only a low percentage of dental plaque bacteria and is involved in the progression of carious lesions and carious dentin is the main ecological site of lactobacilli. They produce either lactate or lactate and acetate from glucose. The most commonly detected species in the oral cavity are L.carri and L.acidophillus.

Current treatment regimes for plaque related diseases are aimed at controlling the bacteria build-up include mechanical removal of plaque with a tooth brush or flour, chemical methods using topically applied solutions and gels such as fluoride and chlorhexidine and development of a vaccine to protect against caries using S.mutans as the immunizing agent. In the case of caries, a more attractive preposition would be to kill the causative organism in site. Nevertheless, the limited access of topical agents to the plaque and the development of antibiotic-resistance create the necessity for alterative strategies to control plaque and to treat gingivitis and periodontal diseases. One such approach is photodynamic therapy (PDT).

PHOTODYNAMIC THERAPY (PDT) is a technique that involves the activation of certain dyes (photosensitizers) by light in the presence of tissue oxygen, resulting in the production of reactive radicals capable of inducing cell death. The main application of PDT

was to treat malignant and some benign[1,66] lesions however, the efficacy of this procedure for microbial reduction has been demonstrated in several studies[6,78,61,64]. This emerging class of applications, which we refer to as antimicrobial photodynamic action (AMPDA), and it shows great potential. Several studies have shown that a large number of oral bacteria, including periodontal pathogenic and cariogenic bacteria, are susceptible to photodynamic therapy[10,16,50,63,65].

Currently, lasers are the most common light sources used to activate the photosensitizers. However, the development of bright light-emitting diodes (LEDs) provides an alternative light source for PDT[36,83,77]. The light produced by an LED has characteristics different from those of laser light. LED is a narrow-band non-coherent type of energy that is not diffraction- limited. In this respect it is similar to the light of a suitably filtered mercury lamp.

LED devices have in general, a lower cost and simpler technology compared to other laser devices, and moreover, they can be readily assembled in several configurations that are suitable for different types of anatomical illumination. However, the use of LED-based light sources to activate photosensitizers for use against oral microorganisms

is a recent development. Study of their use is important to promote the clinical use of LEDs instead of lasers for therapeutic applications in dentistry[84].

The minimal intervention dentistry is a prevailing concept in operative dentistry which addresses that the amount of enamel and dentin should be maximally conserved through the sterilization of cariogenic bacteria, and the stimulation of remineralization[47]. Clinically, the bulk of carious lesion was usually removed by hand instruments or rotary burs, however, the quantity of residual carious dentin to be removed, exhibits great differences among practitioners. A general judgment of residual carious dentin is based on the color by visual inspection and the hardness detected by a sharp excavator[34]. However, this diagnostic criterion is rather subjective and cannot be applied to every dentist.

Caries-disclosing dyes were recommended as an objective method to discriminate the healthy dentin from infected dentin, but the results were not always reliable[8]. In addition, either the hand instruments or rotary burs can not guarantee thorough cleaning of

the infected dentin, or residual bacteria often present before the placement of restoration. Many researchers spend many efforts in developing an optimal method to treat the residual carious dentin.

Photodynamic therapy may emerge as a suitable process to combat both biofilm and antimicrobid - related resistance. Using this technique, a photosensitizer such as haematoporphyrin, phthalocyamine or toluidine blue O **(TB-O)** is activated by irradiation with a light of a specific wavelength (the maximum absorption of the sensitizer) resulting in the generation of cytotoxic species, including singlet oxygen and free radicals which are able to exert a bactericidal effect but which are not toxic to host cells.

The antimicrobial activity is mediated by singlet oxygen, which because of its high chemical reactivity, has a direct effect on extracellular molecules. Thus, the polysaccharides present in EMP of a bacterial biofilms are also susceptible to photodamage. Such dual activity not exhibited by antibiotics represents a significant advantage of photodynamic antimicrobial chemotherapy **(PACT)**. Breaking down biofilms may inhibit plasmid exchange involved in the transfer of antibiotic resistance and disrupt colonization.

In recent years, nanotechnology has permitted the development of new properties of materials. Metal have been used for centuries as bactericidal and bacteriostatic agents. Among those used are silver, gold and zinc, each with different properties and spectrum of activity[4,68,5]. The transition from microparticles to nanoparticles **(<100nm)** in diameter involves an increment in relation to the surface area, among other changes in properties. The antibacterial activity of metal depends on their contact surface a larger surface area of the nanoparticle allows a broader gamut of interactions with other organic and inorganic molecules.

Diverse studies have demonstrated the effect of silver nanoparticles on multiple microorganisms. Many recent studies have revealed that silver nanoparticles have a great bactercidal effect on a several range of microorganism when compared to that of gold and zinc oxide. Its bactericidal effect is known very well and depends on the size and shape of the particle.

The objective of this in vitro study was to isolate selectively the two dominant species of dental caries, streptococcus mutans and lactobacillus acidophillus from the clinical sample of carious dentin. The isolated strains were evaluated for the antimicrobial

effect of photosensitizers activated by LED energy source for decontaminating carious human dentin.

The other aim of the study was to investigate the antimicrobial inhibition activity of the silver nanoparticles exposed for different time intervals to compare the bactericidal effect against S.mutans & L.acidophillus. In addition the antibacterial efficacy was determined using optical density (UV Spectrophotometer) and Environmental Scanning Electron Microscopy (ESEM) examination.

AIMS AND OBJECTIVES

1) To enumerate the bacterial population of the carious dentin.

2) To isolate selectively S.mutans & L.acidophillus from the clinical dentin samples.

3) To observe the antimicrobial effect of LED and nanosilver against S.mutans and L.acidophilus by microbiological methods.

4) To find out the optical density and evaluate the amount of microbial killing using UV-Spectrophotometer

5) To compare the efficiency of LED and nanosilver against the microorganisms and its visualization by ESEM.

Review of Literature

Streptococcus mutans and Lactobacillus acidophilus:

Walter Joesche et al [76](1986) reported a review regarding the role of *Streptococcus mutans* in human dental decay and elicited the understanding of the ecology of *Streptococcus mutans* suggesting that the treatment strategies which interfere with the colonization of *Streptococcus mutans* may have a profound effect on the incidence of dental decay in human populations.

F Elizabeth Martin et al[24] (2002)examined a quantitative microbiological study of human carious dentin by culture and real -time PCR and suggested the presence of high levels of gram positive lactobacilli and also anaerobes such as *Prevotella spp and Fusobacterium* in carious lesions may be indicative of irreversible pulpal pathology.

Munson M A et al [42](2004) studied the molecular analysis of the microflora associated with dental caries and characterized that the bacterial community of the middle and advancing front of carious dental lesions by cultural and molecular analyses.

Boonyanit Thaweboon [9] et al (2006) undertook a study to evaluate the validity of a new modified dip-slide test for the estimation of Salivary mutans streptococci ,lactobacilli and candida levels by comparison with the results obtained from conventional agar plate counts.

Jorn A.Aas et al [29](2008) investigated the bacteria of dental caries in primary and permanent teeth in children and Young adults and concluded that bacterial profiles change with diseased states and differ between primary and secondary dentitions.

Cristian Roman -Mendez et al[13] (2009) reported a study for the identification of oral strains of *Lactobacillus* species isolated from Mexican and French children and found that *L.acidophilus, L.rhamnosus and L.brevis* as potential cariogenic agent.

Photodynamic therapy and Photosensitizers

Burns .T et al [12,11](1993,1994) studied the sensitization of cariogenic bacteria to killing by light from a helium - neon laser and showed a substantial killing rate of the *Streptococcus mutans* was achieved with a dye concentration of 50µg/ml and a light energy dose of 33.6 J/cm^2.Effect of gallium aluminium arsenide diode laser .

David Kessel et al [19](1999) studied the photodynamic therapy and a mitochondrial inducer of apoptosis, showed that photodamage to the mitochondria of murine lurcine P388 cells resulted in immediate loss of the mitochondrial membrane potential together with the release of cytochrome C into the cytosol.

Veruska et al [74](2003) studied the ablative effects of Nd:YAG or Er:YAG laser radiation and showed caries removal were not completely efficient. Depending on the energy density applied both lasers remove not only caries but also healthy tissue and also temperature on the pulp chamber was monitored.

Ulrich Schoop et al[72] (2004) studied the bactericidal effect of different laser systems in deep layers of dentin and demonstrated that all the wavelengths investigated are suitable for the disinfection of even the deeper layers of dentin.

J.A.Williams et al [31](2004) determined the susceptibility to photo-activated disinfection of Toluidine Blue O in a collagen matrix and in carious dentine against *Streptococcus mutans* in vitro and showed that PAD can achieve approximately 99% killing of bacteria.

Pearson G.J. et al [51](2004) used a novel disinfection technique to improve restoration caries and root canals and showed that in dental caries the use of PAD can eliminate residual bacteria in softened dentine and provide an environment which encourages rapid healing.

Ron R Allison M.D. et al [57](2004) studied the use of photosensitizers in clinical PDT and showed photosensitizers in photodynamic therapy allow for the transfer and translation of light energy into a type II chemical reaction. They have also evaluated the potential benefits and the consequences of each major photosensitizer that has been tried in a clinical setting.

Tatiana N. Demidova et al [69](2005) studied the effect of cell-photosensitizer binding and cell density on microbial photoactivation and concluded that the number and mass of the cells compete, both for available dye binding and for extracellulary generated reactive oxygen species.

Tony P.Paulino et al[70] (2005) investigated the photodynamic therapy (PDT) in vitro acting on *Streptococcus mutans* and fibroblasts using hand held photopolymizier (HHP) and a classical photosensitizer (Rose Bengal) for inducing photodynamic response, showed that the

photoactivation of Rose Bengal using the HHP inactivated the bacteria without affecting the fibroblasts viability.

Iriana Carla Junqueire Zanin et al[28] (2005) evaluated the antimicrobial effect of Toludine blue O in combination with either a helium/neon (HeNe) laser on a light emitting diode (LED) on the viability and architecture of *Streptococcus mutans* biofilms

Giusti J.S.M. et al[25] (2006) evaluated the effectiveness of photodynamic therapy on the decontamination of artificially induced carious bovine dentin using photogem as the photosensitizer agent and on LED device as a light source.

Simon Wood et al [62](2006) evaluated the clinical plaques disclosing agent erthyrosine as a photosensitizer in the photodynamic killing of the oral bacterium *Streptococcus mutans* grown as a biofilm and demonstrated that erythrosine to be more effective photosensitizer than the two established photosensitizers methylene blue and photofrin.

Daniel Metcarf et al [14](2006) aimed to increase the bacterial cell killing efficiency of erythrosin mediated photodynamic therapy of *Streptococcus mutans* biofilms by fractionating the delivered light dose into a series of shorter pulses and showed erythrosine mediated PDT of

Streptococcus mutans biofilms can be further enhanced by fractionation of the applied light dose.

Tim Maisch et al [67](2007) investigated the role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria and observed that oxygen supply is a crucial factor in the efficacy of photodynamic inactivation of bacteria and will be of a particular significance in this approach to be used against multi resistant bacteria.

A.D.Khosravi et al [2](2008) investigated the bactericidal effect of low level laser of Gallium-Aluminium-Arsenide on cariogenic species of Streptococcus and Lactobacilli and concluded that the low level laser of Ga-Al-As was effective in significantly reducing the vability of oral cariogenic bacteria in vitro.

Jucaria et al [32](2008) evaluated the antimicrobial photodynamic action on dentin using a light emitting diode light source in the decontamination of carious bovine dentin using two different photosensitizers like hematoporphyrin and Toluidine Blue-O and concluded that the use of LED energy in association with photogem or TBO was effective for bacterial reduction in carious dentin and that the greatest effect on *Streptococcus mutans* and *Lactobacillus acidophilus* was obtained with TBO at 0.1 mg/ml and a dose of 48 J/cm^2.

Zhouhai Zou et al[85] (2008) investigated the effect of photodynamic therapy with hematoporphyrin monomethyl ether (AMME) on the viability of *Streptococcus mutans* cells on biofilms in vitro and concluded that *Streptococcus mutans* cells on biofilms were susceptible to diode laser in the presence of HMME suggesting that this approach may be useful in the treatment of dental plaque related diseases.

Karl Stocec et al[35](2008) studied the effect of smear fiber tips for dental laser applications allowing to increase the quality of laser ablation to modify laser -induced tissue effects significantly and to conceive new indications that are otherwise not possible.

Rene Frazen et al[56] (2009) evaluated the depth of effectiveness of erbium, chromium, ytthium, scadium, gallium, gavent (Er,Cr:YSGG) laser irradiation on microorganism reduction and elicited the low pulse energy of 3.13 KJ, the Er, Cr: YSGG laser irradiation resulted in significant bacterial reduction upto a dentin thickness of 500µm.

Mihaela Antonino Calin et al[40] (2009) studied the light sources for photodynamic inactivation of bacteria using both coherent and non-coherent light sources and also using photosensitizing substances.

Samir Nammour et al[58] (2009) studied to verify the harmlessness for pulp vitality of photo activated decontamination in caries treatment by evaluating the increase in temperature during photo activated disinfection and showed that the dental pulp temperature rise following the use of PAD technique for the decontamination of dentine can be considered as safe procedure for pulp vitality.

Anticariogenicity in non PDT

F.Ozer et al [23](2003) compared the antibacterial activity of two dentin bonding systems (AB7, kuraray and Reactmer Bond, Shofer) by a conventional agar well technique and newly designed invitro test using tooth model and concluded that AB7 has demonstrated to be able to inactivate the bacteria in the cavity effectively in comparison with little antibacterial effect shown by Reactmer bond.

Laurylene Cear de Souza Vasconcelon et al [38](2006) investigated the minimum inhibitory concentration of adherence of Punica grantum Linn (pomegranate) gel against *Streptococcus mutans, Streptococcus mitis and Candida albicans* and the results of this study support the possibility of that the pomegranate gel might be used in the

control of bacteria and yeast responsible for oral infections such as caries ,periodontal disease and somatitis.

Yoshiko Hayashi et al [80](2007) evaluated the chewing chitosan containing gum effectively inhibiting the growth of cariogenic bacteria and suggested that the supplementation of chitosan to gum is an effective method to control the number of cariogenic bacteria, especially for situations difficult to brush one's teeth such as when an individual is away from home or participating in outdoor training.

Tin O M et al [71](2007) examined the antibacterial effect of locally produced hydroxyapatite using *Streptococcus mutans* and showed that bacterial growth inhibition occurs from 50mg/ml to 200mg/ml of HA (complete inhibition).So the the antibacterial property of HA should be used in advantage as a bioactive biomaterial in dental and maxillofacial applications.

Satoshi IMazato et al [59](2008) studied the bactericidal activity of an antibacterial monomer MDPB (12- methacryloloxy dodecylpyridium bromide) against *Streptococcus mutans,* was tested by a rapid method of monitoring cell viability and showed that the staining method thus provided a sensitive means to determine the loss of viability and dictated a strong killing effects of MDPB on *Streptococcus mutans* .

Ella M.Oong et al[21](2008) showed the effects of dental sealants on bacterial level in caries lesions and showed the sealants significantly reduce the bacterial levels in cavity lesions and also support that those of recent analysis that sealants prevented caries progression, When sealants are retained access to fermentable substrates is blocked bacteria do not appear capable of exerting their cariogenic potential.

Yogesh Kumar et al [79](2008) evaluated the antibacterial activity of an herbal dentrifice against *Streptococcus mutans* and *Lactobacillus acidophilus* and concluded that the test dentrifice arodent possess good antibacterial activity against these cariogenic bacteria and this activity is comparable to the standard dentrifice colgate.

Maria de Lourdes et al [48]**(2008)** evaluated the mineral aggregate and calcium hydroxide cement as pulp capping agents in human teeth and shared that the bacteriostatic action of calcium hydroxide and MTA was enough to reduce the number of viable bacteria near the pulp exposure.

Evandro piva et al[22] (2008) studied the influence of a papain based gel (papacarie) for chemomechanical caries removal on bond strength to dentin and showed that the bond strength to carious dentin

of the self -etching system was negatively affected by chemomechanical exaction using the papain based gel.

Yu-Ying Chen et al[81] (2009) investigated that the effects of garlic extract on acid production and growth of *Streptococcus mutans* and showed that despite the stimulation of acid production, garlic may prevent dental caries by stimulating the salivary secretion and inhibition of bacterial growth in the oral cavity.

Silver Nanoparticles

Shizhong Wang et al[60] (2004) studied the use of nanomaterials and singlet oxygen photosensitizers and their potential applications in photodynamictherapy .

Ales Panacek *et.a.* [3](2006) successfully demonstrated the bactericidal effects of size controlled silver nanoparticles using one step modified tollens process. Silver nanoparticles with an average size of 25nm showed high antimicrobial and bactericidal activity against gram +ve and gram –ve bacteria, including highly multiresistant stains.

I Han H et al [27](2007) evaluated the characterization of silver incorporated with calcium phosphate film by RBS and its antimicrobial effects, concluded that higher concentration of silver in the calcium

phosphate film was more effective in reducing the bacteria of *E.coli* and *Streptococcus mutans* on contact with respect to the controls.

Juan Francisco Hernandez-Sierra et al[33](2008) evaluated the antimicrobial sensitivity of *Streptococcus mutans* to nanoparticles of silver,zinc oxide,and gold ,and resulted in that nanoparticles of silver, as compared with those of gold and zinc oxide, require a low concentration to inhibit development of *Streptococcus mutans* strains as low as 5µg/m.

J Kreth et al[30](2008) investigated that the antimicrobial effect of silver ion impregnation endodontic sealer against *Streptococcus mutans* and concluded that silver ions enhance the antimicrobial activity of the root canal sealer against *Streptococcus mutans* using confocal laser scanning microscopy.

Demberelnyamba et al[17] (2008) synthesized silver nanoparticles using hydroxyl functionized ionic liquids and their antimicrobial activity concluding that silver nanoparticles provide environmental friendly and high antimicrobial activity against several gram positive and gram negative bacteria and fungus.

Dhermendra et al[20](2008) investigated the time and dose dependent antimicrobial potential of silvernanoparticles synthesized by

top-down approach and showed the TEM results of binding with bacterial cell membranes are more pronounced against gram negative bacteria compared to gram positive bacteria.

Mritunja et al[45] (2008) in his review reported the antibacterial effect of silver nanoparticles showed that in all the nanomaterials of metallic particles nanoparticles are the best. Nanoparticles increase chemical activity due to crystallographic surfaces with their large surfave to volume ratio.

Dev Kumar Chaterjee et al[15]**(2008)** stated the evolution of nanoparticles in photodynamic therapy,as an emerging paradigm and also telling about the clinical applications of nanoparticles in various field.

Denise Bechet etal[18]**(2008)** studied about the nanoparticles, as vehicles for delivery of photodynamic therapy agents and studied the use of nanoparticles as carriers of photosensitizers .

L.F.Espinosa-Cristobal et al[37]**(2009)** studied the antibacterial effect of silver nanoparticles against *Streptococcus mutans* and concluded that silver nanoparticles present antibacterial activity on *Streptococcus mutans* and this property is better when the particle size is diminished.

I Maliszeoska et al[26](2009) investigated the biological synthesis of silver nanoparticles using fungus mediated synthesis and concluded that TEM micrograph showed the formation nanosilver in the range of 10 -100nm.

MATERIALS AND METHODS

Armamentarium

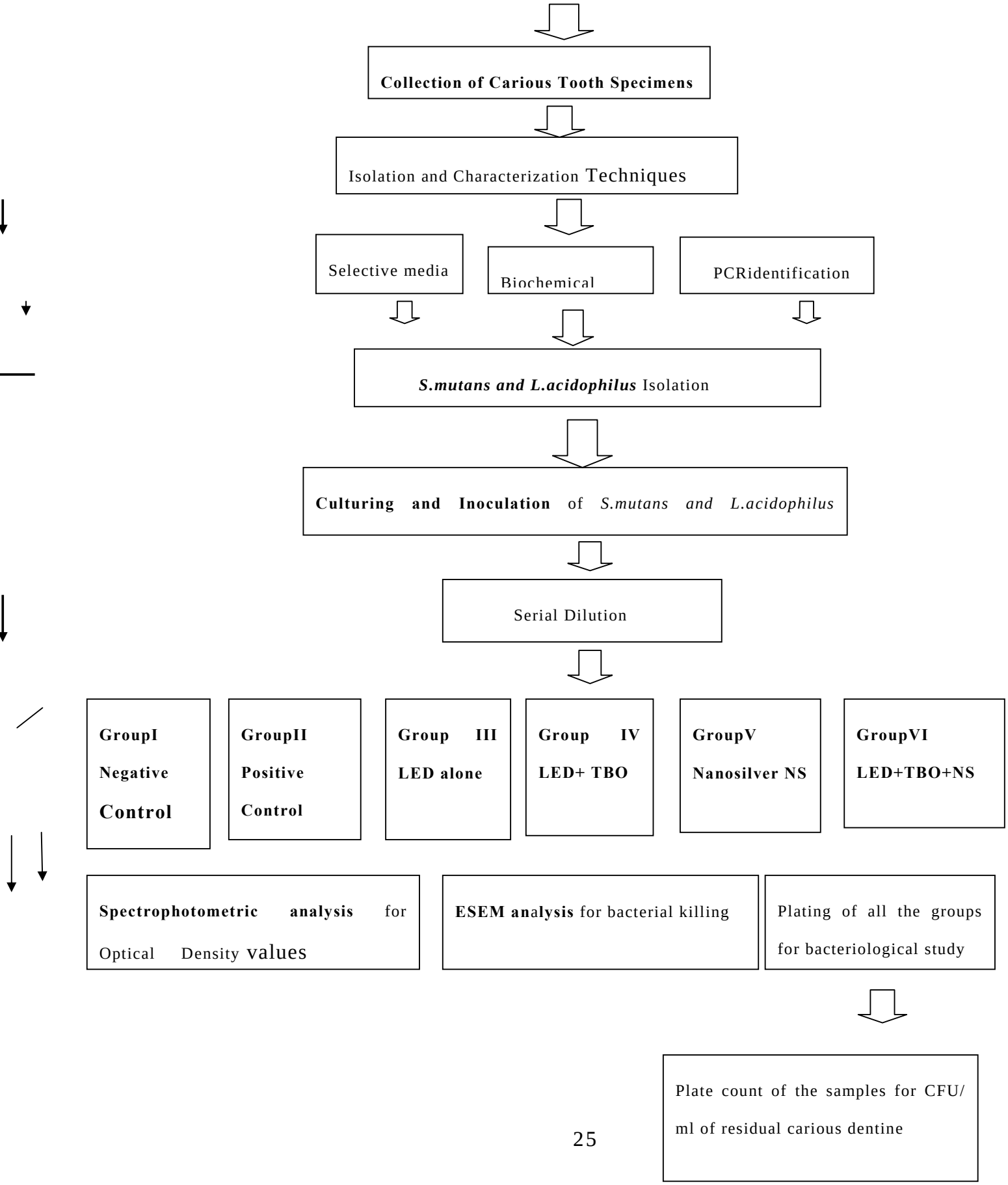
- Himedia- 1. TS agar

  2. Mitis salvarius agar

  3. Rogosa Lactobacilli agar

  4. BHA(Brain heart infusion agar)
- Silver nitrate ($AgNO_3$ – Ranbaxy Lab. Ltd., SAS Nagar, Punjab)
- Sodium tri citrate and Sodium borohydride (99% purity – Aldrich chemicals)
- LED laser Fontana (Zluvania)
- UV – Visible Spectrometer – Cary Model No: 100
- pH meter
- Environmental Scanning Electron Microscopy –FEI Quanta 200 F- edex
- Triple distilled water
- Petri plates
- Stirrer
- Micropipette

METHODOLOGY- FLOW CHART



3.1 Sample Collection:

Twenty four human permanent teeth (16 molars, six premolars and two canines) with primary carious lesions (Class I, classII and class III) were collected from the Department of Oral and Maxillofacial Surgery. Only teeth with lesions that did not involve the pulp were included in the study. Under local anaesthesia, the tooth was extracted and immediately placed in a container of pre-reduced transport fluid (RTF) and transferred to an anaerobic glove chamber containing 85% N_2, 5%CO_2, 10% H_2. Serial dilutions in the range of 10^{-2}-10^{-5} in RTF were prepared for plating.

All the samples were vortexed for thirty seconds using a standardized technique of vertical and rotational movements before plating. Plates were duplicated for each dilution and each medium then incubated in either the glove chamber for up to 2 weeks or for microaerophilic conditions in an anaerobic jar with a CO_2 gas pack (Oxiod, Basingstoke. UK) at 37° C for 48 hours.

3.2. Screening for the Bacterial isolates :(Fig-4.1)

The following media and conditions were used to examine both selective and general growth .Total microbial load was determined by the number of colony forming units **(CFU)** on Trypticase Soy agar under both anaerobic and microaerophilic conditions. Mitis salivarius agar and Rogosa agar were used to study the growth of *Streptococci* and *Lactobacilli* respectively under microaerophilic conditions 95% N_2, 5%CO_2,

3.3. Identification of *Streptococci and Lactobacilli* from other isolates :

Identification is mainly based on morphological and biochemical characterization. An initial group of five specimens were examined to identify the main colony forms on selective media. One colony forming unit of each type was sub cultured, Gram-stained and assessed with biochemical tests. To confirm the accuracy of testing, strains of known species were employed: *Streptococcus mutans* MTCC 890, *Lactobacillus acidophilus* MTCC 497- Imtech ,Chandigarh**(Fig-3)** were used as controls.

3.3.1.Gram staining:(Fig-4.3)

The gram staining techniques were performed to differentiate the gram variety of the above isolates.

The bacterial smears were stained with all these reagents and then observed under microscope to identify their morphological feature. Based on the color of the organisms under microscope they were differentiated into gram positive or negative. The gram positive organisms are stained purple to deep violet color whereas, the gram negative organisms stain red to pink color.

3.2. IMViC test:

The IMViC tests were performed to differentiate the members of Enterobacteriacea family from the other isolates.

3.3.2.1.Indole production test:

The indole production test was performed to determine the production of indole as the end product of tryptophan catabolism . Following incubation of the inoculated media 0.2ml of Kovac's reagent was added and a positive result is indicated by the formation of cherry red colored ring reaction.

The indole production test was performed to determine the production of indole as the end product of tryptophan catabolism. Following incubation 0.2ml of Kovac's reagent was added and observed for the presence of any colour reaction.

3.3.2.2.Methyl red test:

The Methyl red was used to detect the presence of organic acids due to the utilization of glucose as a carbon source. Following incubation of the inoculated media, 5 to 6 drops of methyl red solution was added. Positive result is indicated by the formation of red color.

3.3.2.3.Voges – proskauer test:

The Voges-Proskauer test was performed to detect the production of non-acidic or neutral end products. The test was performed to determine the production of acetoin as an alkaline end product. Following incubation Barrit's reagent was added to the culture tubes. Positive result is indicated by the formation of red color.

3.3.2.4.Citrate utilization test:

The citrate utilization test was performed to check the ability of the organism to utilize citrate as a carbon source. Following incubation, the

tubes were observed for color change from green to Prussian blue which indicates the deposition of acid end product.

3.3.3. Hydrolysis reactions :

The hydrolysis reactions were used to convert high molecular weight polymer to a low molecular weight polymer and are indicative of the special enzymes produced remarkably by the candidate organism.

3.3.3.1.Starch hydrolysis test:

The Starch hydrolysis test was performed to determine the organism's ability for the degradation of starch.

Starch hydrolysis test is performed to determine the starch hydrolyzing activity by the enzyme Amylase. The starch agar plates were inoculated with the culture and kept for incubation. After incubation, the plates were flooded with lugol's iodine solution. The test plates were observed for the presence of zone of hydrolysis.

3.3.3.2. Casein hydrolysis :

The test was done to identify the organism's ability to degrade the milk protein casein. After incubation of the inoculated casinate agar plates for 24 hours, the plates were observed for zone of hydrolysis around the colonies.

3.3.3.3.Esculin hydrolysis:

The Esculin hydrolysis was performed to indicate the organism's ability to degrade protein esculin. The test plates following inoculation and incubation were observed for the production of black zone around the growth of the organism.

3.3.3.4.Gelatin hydrolysis test:

The gelatin hydrolysis test was performed to determine the ability of the organism to degrade gelatin. Nutrient gelatin tubes were prepared and sterilized. The tubes were inoculated with the isolate and incubated at 37°C for 24 to 48 hours.

After incubation the tubes were placed at 4°C for 30 minutes along with a control tube. The tubes were examined for the gelatin hydrolysis in comparison with the control.

3.3.4. Urease test:

The test was performed to determine the organism's ability to utilize urea and convert into ammonia, an alkaline end product by the enzyme urease. Following incubation of the inoculated media, the test tubes were observed for the appearance of pink color.

3.3.5. β – galactosidase (ONPG) test :

The test was performed to determine the organism's ability to catabolise lactose by the production of the enzyme ß-galactosidase enzyme.

The peptone water medium was sterilized at 121°C at 15lbs for 15 minutes. One part of ONPG was dissolved in three parts of peptone water aseptically and the pH was maintained at 7.5.The mixture was distributed in 2ml amounts and incubated for 24hrs to test sterility. The test culture was inoculated in the tube and incubated for 24hrs at 37°C. A positive result was indicated by the development of yellow color.

3.3.6.Oxidase test:

The test was performed to detect the oxidase producing ability of the isolate. The oxidase disc containing the reagent Tetramethyl paraphenylene diamine dihydrochloride was placed on the culture plate. The formation of deep violet color indicated a positive result.

3.3.7. Catalase test:

The catalase test detects the ability of the organism to produce the enzymes catalase or peroxidase which rapidly reacts with hydrogen peroxide.

1ml of 3% Hydrogen peroxide was taken in a clean glass slide. A drop of the culture was mixed with it and examined after 5minutes The drops was observed for the production of gas bubbles.

3.3.8. Hydrogen sulphide production test:

The test was performed to detect the ability of the isolate to produce hydrogen sulphide gas by reducing organic sulphur. Sulphide indole motility agar was prepared and sterilized. The medium was dispensed into sterile test tubes and agar deep was inoculated with test organism to a depth of 1cm. Tubes were incubated at 30°C for at least 4

days.Tubes were observed for both spreading of growth and also for the presence of blackening due to the production of hydrogen sulphide.

3.3.9.Carbohydrate fermentation test:

The Carbohydrate fermentation medium was prepared and inoculated with the test culture. Then the indicator disc was inserted into the inoculated medium aseptically. After incubation the medium was observed for the production of yellow color due to acid production.

3.4 PCR Identification:(Fig-4.2)

3.4.1 Chromosomal DNA Extraction:

The samples 10µl were plated on selective medium. The colonies were collected and stored in TE buffer at -20°C until they were used for the PCR.

Overnight bacterial pure cultures (about 0.5-1 billion Bacterial cells) were collected and suspended in 0.5 ml TE buffer and small scale chromosomal DNA extraction procedure was used. Briefly, cells were washed in 0.1 M Tris buffer, lysed with lysozyme + 10% SDS and extracted with phenolchloroform. DNA was precipitated with sodium acetate and ethanol, and its concentration was calculated by measuring absorbance at 260nm and 280nm using a UV-spectrophotometer.

3.4.2 Oligonucleotide Primers and PCR procedure:

3.4.2.1 PCR Primers:

The following universal primers were used:

1. Universal primers for *streptococci* (degenerative primers) **(Poyart et al., 1998)** to amplify a 480-bp fragment.

D1: 5'-CCI TAY ICI TAY GAY GCI YTI GAR CC-3'

D2: 5'-ARR TAR TAI GCR TGY TCC CAI ACR TC-3'

2. Universal primers for *Lactobacilli* (degenerative primers) **(Sul,Su-yeon et al., 2006)** to amplify a 575-bp fragment.

D1: 5'-CAC TTC GGT GAT GAC GCI GTT GG-3'

D2: 5'-CGA TGC AGT TCC TCG GTT AAG CT-3'

The following PCR reaction mixture was used as that of **(Oho et al 2000):**

PCR reaction mixture (25µl):

12.5 µl Hot StarTaq Master Mix,0.25 µl primer D1,0.25 µl primer D2,11 µl distilled water,1 µl DNA

PCR program:

95°C for 15 min

95°C for 30 sec (denaturation) } 40 cycles of amplification

37°C for 30 sec (annealing)

72°C for 90 sec (elongation)

72°C for 10 min

4°C storage

The PCR conditions were denaturation at 95°C for 30 seconds , followed by annealing at 37°C for 30 seconds and extension at 72°C for 90 seconds. This amplification was repeated for 40 cycles. The final cycle was run at 72°C for 10 minutes. Along with these samples, positive and negative controls were run for each experiment, using purified genomic DNA from *S.mutans* **(MTCC 890)** and *L.acidophilus* **(MTCC497)** respectively. As positive controls and distilled water as negative controls. PCR products were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide.The resulting bands were viewed in UV light and photographed with a digital camera.

3.5 Preparation of Dentin Specimen:(Fig-4.4)

Extracted human third permanent molars were used in this study . Crowns with caries, restorations, or fractures were discarded. Any remaining soft tissues were thoroughly removed from the tooth surfaces with a dental scaler (Sonicflex 2000, KaVo Co., Biberbach, Germany) under running water. All teeth were then stored in 48°C distilled water containing 0.2% thymol to inhibit microbial growth until use. While fully hydrated, each third molar was first cut just below the occlusal pit and fissure, perpendicular to the long axis of the tooth by means of a hard tissue microtome **(**Fig**3.10)**. Second, the crown dentin disks with thickness of 1 mm were obtained by a second cut horizontal to the first one.

The enamel of each specimen was removed with a plain-cut tungsten carbide fissure bur at high speed under continuous water spray. Each dentin disk was subsequently cut to an orthorhombic shape with the dimensions of length x width 9x7mm. Then the dentin specimens were wet-polished with a 600 grit silica paper to create uniform flat surfaces and to finely adjust the specimen thickness. The specimen thickness was precisely determined by an electronic vernier.

To ensure complete removal of the smear layer, the specimens were immersed in an ultrasonic cleaner filled with 10% ethylenediaminetetraacetic acid **(EDTA)** for 2 minutes and 2.5% sodium hypochlorite **(NaOCl)** for 1 minute, followed by three washes with physiological saline solution for a period of 2 minutes.

3.6 Photosensitizer and Laser Irradiation:(Fig4.7)

A diode laser that provided a constant beam of coherent, continuous monochromatic light with an emission wavelength of 810 nm was used in this study. A light-emitting diode **(LED) (810nm, 1W)** was used as an aiming device and the laser beam was delivered through a flexible **300µm** optic fiber with a straight handpiece in pulse mode. Before the laser irradiation, the laser energy was carefully calibrated with a power meter to control the output energy from the fiber tip within the desired irradiation condition.

The calibration of laser energy with a power meter after laser irradiation was also performed. The results showed that the laser energy output was the same as our desired irradiation parameters. The laser tip was held perpendicular to the irradiated surface in a light contact motion and was cut **1 mm** after irradiating each specimen to prevent energy loss. It was swept with the whole irradiation area of 9 mmx7

mm at a speed of about **1 mm/sec** with a total irradiation time of **60 sec** to simulate clinical manipulation.

Toludine blue O **(Sigma-Ahlrich,Germany)** solution was freshly prepared at a concentration of **0.1mg/ml** by dissolving in distilled water at a pH of **5**.

3.7 Synthesis of Nanosilver Solution:(Fig-4.9)

A solution containing **100µg** of silver nanoparticles in 1ml was prepared by adding 5 ml of 10^{-4} M solution of silver nitrate with 5 ml of 0.1M sodium tricitrate. The solution was brought to a higher temperature about 45°C and stirred well. Then a few drops of 0.1 M sodiumborohydride ($NaBH_4$) was added until the colour of the solution changed from colorless to yellow. While adding sodium borohydride the solution was stirred very fast. After 30 minutes the temperature was lowered to room temperature and then the solution was left with stirrer for the growth process of homogenized sized silver nanoparticles.

3.8 Test groups:

Sixty dentin specimens each with 1mm thickness were used for this experimentally study. The specimens were randomly divided into

six groups G-I to G-VI with ten specimens in each group. The following treatments and conditions were tested in each group.

Group I - Negative control

Group II- Positive control

Group III-LED Laser Treatment

Group IV-LED Laser + TBO Dye Treatment

Group V -Nanosilver treatment at different time intervals of 20, 40, 60, 80 seconds

Group VI-Dye + Nanosilver + LED treatment

3.9 Microbiological Processing: (Fig-5)

3.9.1 Inoculum and Media:

The microorganisms used in this study were *S.mutans* and *L.acidophilus* isolated from carious tooth. To prepare the inoculum the isolated strains of *S.mutans* and *L.acidophilus* were grown anaerobically on Brain -heart infusion (BHI) agar and MRS Agar plates respectively for 3 days. Subsequently, single colonies were inoculated into 10 ml of BHI broth and incubated anaerobically at 37°C overnight.

3.9.2 Bacterial Inoculation:

All specimens of the experimental groups were autoclaved for 20 minutes at 121°C and transferred to individual test tubes, each containing 5 ml of a brain heart infusion **(BHI)** culture medium supplemented with 1g/100ml of glucose and 2g/ml of sucrose. For each 50 ml of medium solution, 5ml of 10^6CFU/ml *Lactobacillus acidophilus* and 5ml of 10^6 CFU/ml of *Streptococcus mutans* were added to induce bacterial colonization. The test tubes were maintained in a microaerobic environment at 37°C for 10 days, with the solution changed every 48 hours. Following the 10-day period, the specimens were maintained under refrigeration at 4°C until treatment.

The control Group I was used as negative control containing only the dentin sample.

The control Group II was only inoculated with 5 ml of *S. mutans* and *L.acidophilus* but without receiving any treatment.

The specimens of experimental Group III were irradiated with a light-emitting diode **(LED)** delivered through a flexible **300µm** optic fibre with wavelength centered at **810 nm**.The generated light was collimated to a spot size of **0.8 cm** in diameter. The total irradiance was **1W/cm^2** and the exposure time was **60 sec**.

The specimens of experimental Group IV were investigated for the action of a photosensitizer TB-O. The specimens were immersed in a solution containing **0.1mg/ml** of TB-O for about **60 sec**. Then the specimens were irradiated with a light-emitting diode (LED) of **1W/cm^2** and the exposure time was **60 sec** to study the influence of both dye and LED on bacterial inhibition.

The specimens of experimental Group V were investigated for the antibacterial action of silver nanoparticles. The specimens were immersed in a solution containing 100µg/ml of nanosilver for different time intervals of about **20,40, 60**and **80** seconds.

The specimens of experimental Group VI were investigated to find the influence of LED, dye and nanosilver **(100µg/ml)** for the antimicrobial action. The specimens were first immersed in nanosilver solution **(20sec)** followed by immersing in 0.1mg/ml of TBO for about **(60 sec)** and finally LED laser treatment was given.

3.9.3 Bactericidal Evaluation:

After treatment the specimens were placed into individual test tubes with 5 ml of saline solution. Each tube was then vortexed for

1 minute to remove the bacteria from the dentin surfaces. Upon vortexing, 0.1 ml of the extracted fluid was diluted in log 10 steps. One hundred micro liters portions of the dilutions ($10^{-}1$ to $10^{-}5$) were applied to the BHI culture plates and incubated for 48 hours at 37^{0}C anaerobically. The colonies were then counted using a digital counter and the total number of bacteria (colony forming units (CFU) per/ml) was assessed. The number of CFU was divided by the weight of each sample to obtain the number of CFU per milligram (CFU/mg) of the carious dentine.

The results were quoted in ‘‘percentage of killing’’ as:

Percentage of killing = [100- (CFU of experiment group/ CFU of control group) x 100]

3.9.4 Determination of Microbial Density:(Fig-3.5)

Three specimens in each group were used to evaluate the bacterial cell mass by measuring the absorbance values at 540 nm. All the test groups were compared of their bactericidal inhibition by measuring the cell density with the help of **UV- spectrophotometer**. After treatment the specimens were placed into individual test tubes with 5 ml of saline solution. Each tube was then vortexed for **1** minute

to remove the bacteria from the dentin surfaces and their optical density values were measured at **540 nm** and noted.

3.9.5 ESEM Analysis:(Fig-3.9)

Specimens in each group were used to perform morphological examination of surface of dentin by ESEM **(Environmental Scanning Electron Microscope)**. The control group specimens were also visualized to see the surface of the dentin.

3.9.6 Statistical Analysis:

Non-parametric statistical methods were applied to determine the significance of the presence of laser alone, the combination of photosensitizer and laser irradiation, the data was analysed by a variance analysis (ANOVA) model using the factorial (2x2) design. The Turkey test was chosen for evaluating the significance of all pair wise comparisons with a significance limit of 5%.

Results:

Bacteria Cultured from carious dentine samples:

The number of CFU per milligram of carious dentine showed considerable variability, as did the specific genera detected in individual samples and showed considerable diversity of the microflora, as showed in **Table 4.1**. Hundredfold difference in total microbial loads were apparent between samples, while the loads of specific genera and species in the carious dentine varied upto three orders of magnitude between individual teeth.

Data for selective microbial groups were noticeably skewed, with the mean values greater than the median values in each case. Anaerobic microorganisms were isolated from all samples and the numbers of colonies cultivated anaerobically, on non-selective plates were approximately six times greater than those grown under microaerophilic conditions. Analysis of the numbers of CFU showed a

predominance of gram-positive bacteria, with *Lactobacillus* spp. being cultivated in the greatest numbers on the selective media. Gram - negative organisms were also present in significant numbers such as *Prevotella* spp., and Actinomycetes, being the largest of the anaerobic group were not considered for this study.

Two main colony types were distinguishable on mitis salivarius agar. Biochemically these resembled *S. mutans and S.sobrinus.S.mutans* strains formed mulberry-shaped colonies growing deep into the agar, while the very hard colonies of *S.sobrinus* were usually surrounded by extracellular polysaccharides. *Lactococcus, Enterococcus* species were also present. *Lactobacilli* were grown on Rogosa agar and four main colony forms were identifiable. These resembled *L.acidophilus, L.rhamnosus ,L. fermentum and L.plantarum* when tested biochemically. *Lactobacillus acidophilus* presented in different colony configurations was found numerically dominant.

Table 4.1 Bacteria detected in carious dentine by colony counting

Bacterium	No of CFU (mg/ dentine)*		
	Range	Median	Mean ±SEM
Anaerobic	$1.3x10^5$-$3.7x10^7$	$5.2x10^6$	$(7.1±0.7)x10^6$
Microaerophilic	$2.1x10^4$-$4.4x10^6$	$8.2x10^5$	$(1.3±0.1)x10^6$
Streptococci	0.0-($3.2x10^6$)	$1.7x10^5$	$(3.6±0.5)x10^5$

Lactobacilli	0.0-($1.7x10^7$)	$5.1x10^5$	$(1.0\pm0.5)x10^6$
Prevotella	0.0-($4.9x10^6$)	$1.0x10^5$	$(4.6\pm1.3)x10^5$
Actinomycetes	0.0-($5.2x10^6$)	$7.5x10^4$	$(1.7\pm0.4)x10^5$

*** Data collected from 24 samples**

Colony counting showed that of the 15 carious teeth, 13 (97%) were positive for *Streptococcus* spp., and 12 (95%) were positive for *Lactobacillus* ssp., and the remaining nearly 91% is of other species such as *Prevotella* and *Actinomycetes.*

The use of biochemical tests to identify microorganisms from dentine caries provided variable results and indicated that microbes of different appearance could be identified as the same organism. The PCR detection of *S. mutans and L.acidophillus* was achieved in minimum time with the help of universal probe confirmed with the reference strains as shown in the **Fig- 3**.

Table 4.2 Biochemical characterization of the selective isolates

Biochemical characters	Isolates of Streptococci and Lactobacilli					
	S.mutans	S.sobrinus	L.acidophilus	L.rhamnosus	L.fermentum	L.plantarum
Gram staining	+	+	+	+	+	+
Morphology	spherical	Rods	rods, pairs, chains	Curved rod	Straight rods,pairs	Curved rods
Motility	-	-	-	-	-	-
Catalase	-	-	-	-	+	+
Oxidase	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+
Indole	+	+	+	+	+	+
H2S	-	-	+	+	-	ND
ONPG	-	ND	+	-	+	ND
Starch	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
Growth at 40°C	-	-	+	+	+	+
Gelatinase	-	ND	+	+	+	+
Urease	-	+	+	+	+	+
Maltose	+	+	+	+	+	+

Mannitol	+	+	+	+	+	+
Sorbitol	+	ND	+	+	+	+
Raffinose	+		+	+	-	ND
Sucrose	+	+	+	+	+	+
Lactose	+	+	+	+	ND	+
4%Nacl	-	-	+	+	+	+
Citrate	+	-	-	-	+	ND

+ - Positive; - - Negative; ND- Not determined

The antimicrobial photodynamic action the diode laser used in this study was evaluated by bacterial enumeration of colony forming units and it is represented by Percentage of bacterial inhibition or killing. This expression may represent a simple tool to evaluate the efficacy of antimicrobial photodynamic action of different experimental test groups against the cariogenic bacteria. The results are shown below.

Table4.3 Effect of Bactericidal Efficiency of Diode Laser in different Test Groups :

Bacterial strain	**Contact time**	**Energy dose**	**Groups**	**Antimicrobial treatment methods**	**CFU/ml (x±SD)**	**Percentage of Killing**
S.mutans and L.acidophilus	**60 sec**	**48 joules**	**G-II Control**	**Nil**	**$218\pm13x10^5$**	-
			G-III	**LED**	**$135\pm18x10^5$**	**40%**

			G-IV	LED+ TBO	48±x10^{5*}	82%
			G-VI	LED+TBO+ NS	0.00*	100%

***Represent values that are statisticaly significant (P<0.05)**

Group V specimens tested upon the antimicrobial action of nanosilver showed considerable bactericidal efficiency when the exposure in the nanosilver solution was increased constitutively. It was interesting that no colony was formed in the specimen immersed in nanosilver solution for more than 60 seconds, that gave almost a 100 % of antibacterial action. The results are shown below. Table and fig 4.4

Table 4.4 Effect of Nanosilver of Bacterial inhibition on different time intervals:

Concentration	Time of exposure seconds	Bacterial inhibition CFU/mg	Percentage of killing
100µg/ml	20	125 x10^{5}	38%
100µg/ml	40	76 x10^{5}	56%
100µg/ml	60	41x10^{5*}	87%
100µg/ml	80	0.00*	100%

* **Represent values that are statisticaly significantl (P<0.05)**

All the specimens of the test groups were measured for their microbial density and cell mass by UV- spectrophotometer and their optical density values were given in the table below.

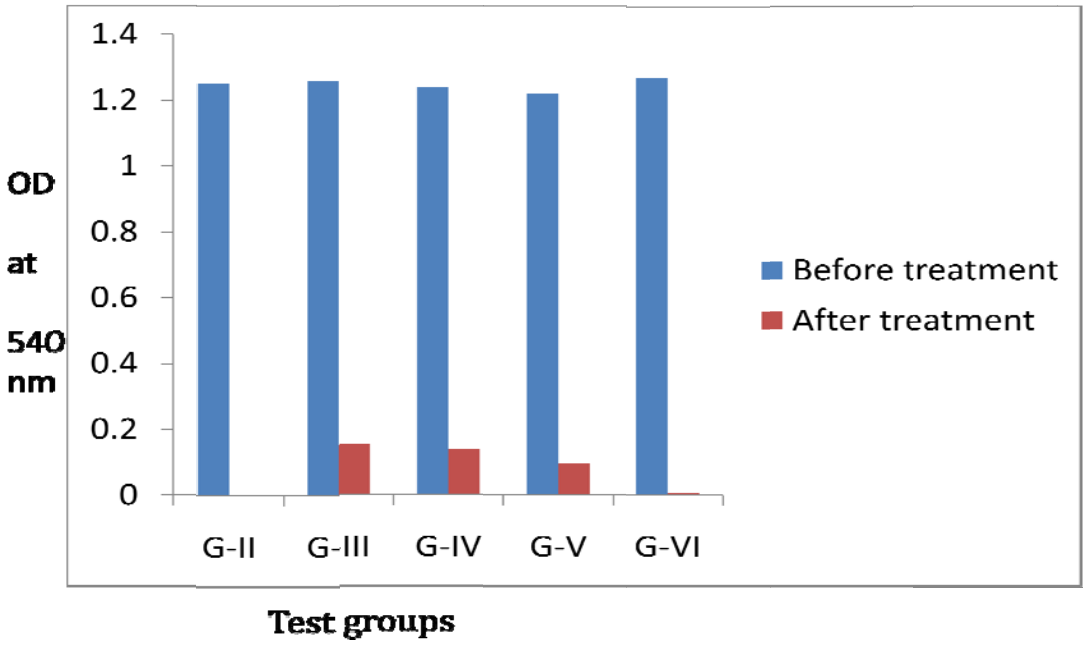
Table 4.5 Determination of Optical density values in the test groups:

Test groups	Mean Optical density values	
	Before treatment	After treatment
G-II Control	1.25	-
G-III	1.26	0.156
G-IV	1.24	0.139
G-VI	1.22	0.098

ESEM Examination:(Fig 5.6-Fig 6.1)

All dentin specimens without laser irradiation Group-I (negative control group) demonstrated even and clean surfaces showing exposed dentinal orifices and there were no smear layer noted.Group -II (positive control) showing full growth of microbes by forming a thick biofilm and also the microbes penetrating into the dentinal tubules .After the treatment of LED laser (group- III) exposed dentinal tubules with moderate microflora was observed and there is decrease in the penetration of microbes into the dentinal tubules.. Group IV showed significant reduction of bacterial adherence on dentinal tubules compared to Group- III and there is a thin lining of biofilm without microbial penetration has seen. Group V and Group VI demonstrated similar microstructures as illustrated in the Figures 4.10 without any bacterial colonization and the presence of prominent and o dentinal tubule orifices,except in Group- V shows nanosilver into the dentinal tubules.Group- VI shows clean surface without microbial growth and biofilm layer,showing exposed dentinal tubules as like Group-I.

Fig.4.5 Effective of Different treatments on Microbial density



Discussion:

Dental caries is an infectious disease in which members of the *S.mutans* group have been implicated as major etiological agents. *S.mutans* and *S.sobrinus* are the most commonly found species in humans and the correct identification and differentiation among them is an important step to understand the early phases of colonization of the oral cavity. In the present study, biochemical tests allowed the identification of *Streptococci* and *Lactobacilli* species in carious affected samples in good agreement with PCR results and with the help of reference strains from MTCC, Chandigarh **(Fig-3)**

The results of the present study are in agreement with others in a finding a large variation in microbial counts between samples. This study also confirm that the largest group of isolates from carious dentine is the facultative gram-positive rods, even though *Lactobacillus* spp were isolated in lower frequency .The finding of significant numbers of gram - negative anaerobic bacteria, has also been reported

Prevalence of the Gram-positive microaerophilic bacteria following culture of the carious dentine may be a result of their high numbers or due to the relative ease with which these microbes can be cultivated compared with Gram-negative

anaerobes. During cultivation of the carious samples a number of in consistencies were noted as such of great variation in colony morphology and biochemical identification. The present research was narrowed down for the identification of species of only *Streptococci* and *Lactobacilli* by the use of an appropriate molecular method of bacterial identification through PCR technology with the help of universal primers against the reference strain gave a satisfactory result for the identification of both the *Streptococci* and *Lactobacilli* species. The rest of the bacteria identified were not considered further.

Streptococcus mutans were selected in this study because they were the predominant bacteria in carious lesions[73] .Compared with gram negative bacteria, it is relatively resistant to laser irradiation because of their tough cell wall, which is composed of highly cross-linked murein[44].

Several studies have shown that oral bacteria are susceptible to PDT when they are grown as planktonic cultures[52] and .Photodynamic action has been used to kill oral microorganisms since the beginning of 1990's, when studies demonstrated that some photosensitizers show an affinity for bacterial walls and can be photoactivated to cause the desired damage.

Ito, in 1977 showed that TBO was photodynamically active, promoting yeast cell death without inducing any genetic alterations, and that the cellular membrane was the reaction site for singlet oxygen. The effect on the yeast cells was to increase permeability and loss of control over permeability, resulting in an imbalance of intracellular substances, resulting in cellular death without any apparent chromosomal damage.

Paardekooper et al[53] observed that toluidine blue entered into cells during illumination because of a sudden change in the cellular membrane, resulting in damage to the cellular membrane and intracellular structures. Studies of biofilms have shown the efficacy of TBO at concentrations of 0.01% and 0.1% when illuminated with HeNe laser energy, for reduction of the numbers of *Streptococcus sanguis, Porphyromonas gingivalis, Actinobacillus, Actinomycetes and Fusobacterium nucleatum.* An apparent bactericidal effect was observed after exposure to laser energy for 30 sec, with a light dose of 219 mJ and fluence of 16.5 J/cm2.

Burns et al.[12] observed that when a suspension of cariogenic bacteria (*Streptococcus mutans, S. sobrinus, Lactobacillus casei* and *Actinomyces viscosus*) was mixed with TBO at 50 μg/mL and exposed

to 7.3 mW of HeNe laser energy, a considerable amount of cellular death was accomplished with a fluence of 33.6 J/cm2.

Okamoto et al.[50] also reported the death of various species of *Streptococcus* using a HeNe laser dose of 720 mJ and a fluence of 5.7 J/cm2 with TBO at 7.5 µg/mL.

Zanin et al [84] evaluated the antimicrobial effect of TBO in combination with either a HeNe laser or a LED, on the viability and architecture of *Streptococcus mutans* biofilms and observed that the bactericidal effect was light dose-dependent,an effect we also observed in this study. The reductions in viability up to 99.99% with both light sources were observed using energy densities between 49 and 294 J/cm2, a pre-irradiation time of 5 min, and TBO concentration of 100 mg/L.

Venezio et al.[75] previously demonstrated that hematoporphyrin derivatives show a bactericidal effect on *S. mutans* and other microorganisms. Gram-negative bacteria seem to be more resistant to this treatment, probably due to their more complex cell wall .Many studies have shown TBO's effectiveness as a photosensitizer, but were performed with bacteria in suspension. However, similar studies demonstrated that the reduction of *S. mutans* in carious dentin

was less than that seen in suspension, and was also less than that attained in a collagen matrix. In that study, it was concluded that the time of contact between the bacteria and TBO was a critical factor.

Williams et al.[77] noted 100% death of *S. mutans* in a planktonic suspension, using a diode laser emitting at 633 ± 2 nm with fluences ranging from 0.4–4.8 J with TBO as the photosensitizer

Those results are similar to the results found in our study, although the presence of the biofilm on the dentin itself negatively affect the efficacy of the technique, because the success of the process depends on photosensitizer diffusion throughout the dentin, and on light penetration and scattering in the tissue. The experimental results of this study indicate that there was bacterial reduction at all investigated parameters using the photosensitizer TBO and its combination with LED in Group III and Group IV for treatment of S. *mutans* and *L. acidophilus*. It has to be noted that, the general effect of toxicity with the exposure to light and TBO increases the bactericidal efficiency nearly 60% when compared with that of laser alone in Group-III giving a very meagre level of Percentage inhibition approximately of 40%. In addition, it is apparent that light alone, without any photosensitizer, has low bactericidal effect.

In this sense, much effort is being devoted to the use of metal nanoparticles in diagnostics, therapeutics and material sciences. Silver nano particles (Ag NPs) used in this study has attracted much attention recently due to their important applications in the fields of nanoscience and biology. Among all these aspects, the use of silver nano particles as bactericidal agents is a field of great interest

Silver has a significant potential for wide range of biological applications as an antifungal agent, antibacterial agent and antiviral for a wide variety of antibiotic resistant bacteria, preventing infections, healing wounds, and anti-inflammatory properties. Although they exhibit strong biocidal effects, they have low toxicity towards human cells and hence its use in this study can be justified.

According to **Ales Panacek *et.al.*[3]** Colloidal silver nano particles with narrow size distribution of 25nm showed antibacterial and bactericidal activity against gram +ve and gram –ve bacteria including highly multi resistant strains such as methicillin resistant *Staphylococcus aureus*, methicillin resistant coagulase negative staphylococcus, vankomycin resistant *Enterococcus faecium*, *Klebsiellae pneumoniae* at a very low concentration of silver like 2μg/ml.

In a study by **Eduardo J. Fernandez *et. al.*[22]** the MIC and MBC of Ag nano particles tested against *E. coli* was 12.5 μg/ml, whereas the MIC against gram +ve *Staphylococcus aureus* was 12.5 μg/ml, and the MBC was 25 μg/ml with the size of Ag nano particles being 10 nm**.** Yet another study by **Ales Panacek[3]** showed Ag nano particles of 25nm size and in concentration of 2 μgm/ml showed antimicrobial and bactericidal property. This study demonstrated 100 μgm/ml of Ag nano particle with a size of 15nm were effective against gram positive bacteria ***S.mutans*** *and* ***L.acidophilus*** at the end of 24 hours.

Various studies have suggested that Silver / Gold nanoparticles may act as an anchor point for light activated antimicrobial treatment and increase its potency. A study by **Jesus Jil Thomas** has shown that Toluidine Blue – O – Troponin coupled gold nanoparticles formed an exceptionally potent antimicrobial agent when activated by white light or 632 nm laser light. However this study was a preliminary step in integrating nano silver based antibacterial effects to optimize thermal based laser treatment. This study was an extension of the previous studies whether the same could be applicable for silver nanoparticles.

It has also been stated that smaller the size of nanoparticles, greater will be its surface to volume ratio and hence greater chemical reacting & biological activity. The same leads to decreased production of reactive oxygen species and free radicals. This may lead to increased oxidative stress, inflammation, consequent damage to proteins, changes in membrane and DNA [45].

In this study silver nano particles were synthesized by borohydride reduction of silver in the presence of citrate. Determination of mean structure, size, distribution, shape is crucial due to the following reasons.

1. Antibacterial properties of silver nano particles are attributed to their total surface area and larger surface to volume ratio provides more efficient means of enhanced antibacterial activity.
2. The nano particles undergo a shape dependant interaction with bacteria.[60]
3. Good dispersion of nano particles is required for effective antibacterial properties.

The plate count at the end of 60 seconds of silver nano particles Group V treated residual carious dentin showed no growth of

bacteria demonstrating superior bactericidal effect of silver nano particles almost to that of Group VI when compared.

Studies by **Ales Panacek**[3] also state that various concentrations of silver nanoparticles required for growth inhibition of bacteria are determined by the biological properties of the individual species. These variations might be attributed to the variation in size of silver nano and the variation in methods adopted for synthesis and other variables.

In this study, the initial decrease in optical density values was observed at about 2hrs. Pour plate done at the end of five hours revealed no growth at the end of 24 hours. In a study by **Eduardo et al** the time taken by various concentrations of silver nanoparticles against representative species was 2.5 hours against gram–ve organisms (very fast) and 7.5 hours against gram +ve organism.

The time required for initiation and killing might have been due to the structural differences between the gram +ve and gram –ve bacteria. The gram –ve have a layer of lipopolysaccharide at the exterior followed underneath by a thin layer of peptidoglycan (about 7 – 8 nm). Although the lipopolysaccharides are composed of covalently linked lipids and polysaccharides, they lack strength and rigidity.

Negative charges on the lipopolysaccharides are attracted towards weak positive charges of silver nano particles. On the other hand gram +ve bacteria are composed of a thick layer of about 20 – 80 nm of peptidoglycon consisting of linear polysaccharide chains cross linked by short peptides to form a three dimensional rigid structure. The rigidity and extended cross linking not only endow the cell walls with fewer anchoring site for silver nano particles but also make them difficult to penetrate. However the extent of inhibition was dependant on the concentration of the nano particles.

Electron microscopic studies done for group II positive control in a time equivalent to irradiation of group III, IV,V and VI demonstrated killing of *S.mutans and L.acidophilus* with a considerable level of decrease in the bacterial accumulation on the dentinal surface. It was so obvious that the disinfected residual carious dentinal surface of Group VI were similar to that of Negative control Group I without any bacterial biofilm accumulation and the dentinal tubules were evenly and clearly visible which might also infer the minimal risk of thermal damage to the pulp. This is in accordance with that of the study reported **by Bor-Shiunn Lee[7] et al** studied the bactericidal effects of diode laser of 7 W on *S.mutans* after irradiation through different thickness of dentin. In this study 7 W was selected as the highest power

to proceed the evaluation of bactericidal effect. The ultimate effect of laser irradiation on tissue depends on the distribution of energy deposited in the tissue, the subsequent heat conduction, and the resultant temperature rise.

Hence this study attempted to

1. Demonstrate the bactericidal effect of LED alone against the common dentinal carious isolate ***S.mutans*** *and* ***L.acidophilus***,Group III
2. Demonstrate the bactericidal effect of LED in conjunction with a photosensitizer TBO against ***S.mutans*** *and* ***L.acidophilus***,Group IV
3. Demonstrate the bactericidal effect of Silver nanoparticles against ***S.mutans*** *and* ***L.acidophilus*** on different time intervals, Group V
4. Demonstrate the bactericidal effect of LED in conjunction with a photosensitizer TB-O and also with Silver nanoparticles against ***S.mutans*** *and* ***L.acidophilus***,Group VI

This PDT technique appears to be replacement for the dentist's drill, producing at least equivalent, if not better, results with the promise of painless treatment. This result is extremely

promising as it suggests that the proposed technique for caries removal preserves healthy dentine and enamel. It was also concluded that the longer contact time was more effective and that optimization of contact between bacteria and dye was an important parameter. Under the best conditions 98% kills were obtained.

The fact that TB-O can sensitize cariogenic bacteria to killing by low power laser light may have clinical implications and in this regard bacteria on the cavity walls and floor could be killed after minimal cavity preparation prior to restoration of the carious lesion. Bacteria in the partially demineralized tissue may also be killed, the possible result of which may be that the amount of diseased tooth tissue requiring to be removed may be reduced.

Although the results of this preliminary study are encouraging, further in-vitro studies are needed to determine whether lethal photosensitization of cariogenic bacteria is possible under conditions more closely resembling those encountered in the carious lesion in vitro where intervening partially decalcified tissue may reduce its effectiveness.

Also the efficacy of the diode laser in disinfecting residual carious dentin needs to be further investigated as the causative bacteria

in carious lesions are not limited to one species and these different species of bacteria might have different susceptibility to the laser irradiation.

Also this experimental study revealed two similar results on the use of LED and nanosilver particles in combination with the photosensitizer to be more effective in bacterial elimination, and also demonstrated the same on longer time contact of Silver nanoparticles to give the more pronounced bactericidal effect much comparable to that of laser and dye. However, further studies must be conducted to verify if the bacteria develop resistance towards the nano particles and to examine the cytotoxicity of nano particles towards human cells before proposing their therapeutic use.

Therefore the future perspectives of this study should address for a more in-depth examination to assess the presence and correlations between bacteria in carious lesions and for the improvement of better strategies to represent a simple tool to evaluate the efficacy of antimicrobial photodynamic action (APDA) for different microorganism species and the use of active or second generation PDT nanoparticles as vehicles for delivery in photodynamic therapy.

Summary:

- Twenty four carious tooth were collected and screened for the isolation and identification of *S.mutans* and *L.acidophillus*.
- The bacteria isolated were investigated for an experimental study of in-vitro antimicrobial photodynamic therapy effect in carious dentin slabs.
- Silver nano particles in a concentration of 100µg/ml were prepared by borohydride reduction method and examined for different time of contact with the infected dentine.
- The experiment was such that 60 slabs of occlusal human dentin were randomly assigned to six groups, exposed to a light emiting diode laser at 630 nm, and with a photosensitizer TBO-100µg/ml and also tested with Nanosilver particles and the combination of all the above antimicrobial treatments as follows:

    1. Group I - Negative control
    2. Group II- Positive control
    3. Group III-LED Laser Treatment
    4. Group IV-LED Laser + TBO Dye Treatment

5. Group V -Nanosilver Treatment at different time intervals of 20, 40, 60, 80 seconds

6. Group VI-Dye + Nanosilver + LED Treatment

- The slabs were subjected to a model for caries production with *S.mutans* and *L.acidophilus* inoculation in Brain heart infusion BHI broth.
- After a 10 -day period of incubation, samples of carious dentin were collected before and after each treatment and cultured for *streptococci mutans* and *Lactobacillus acidophillus.*
- The lighted groups were irradiated with energy density of 48J/cm^2 obtained for about 60 seconds respectively.TB-O was applied for 5 minutes.
- The samples of Group V were immersed in nanosilver solution for 20, 40, 60 and 80 seconds respectively and examined for *streptococci* and *lactobacilli* counts.
- The data were analyzed statistically by ANOVA which showed a significant differences ($p<0.05$) in the groups V and VI of *S.mutans* and *lactobacilli* viability between counts and percentage killing of *streptococci mutans* and *lactobacilli.*

- All the groups Group II to Group VI were subjected to spectrophotometric analysis in time interval of ½ hour and the Optical Density values were measured where an initial decrease in optical density was noted.
- After irradiation, samples from group I to VI were subjected to ESEM analysis. ESEM observation of the residual dentin showed a significant reduction of bacteria deposited on the dentinal surface.

Conclusion:

Owing to the emergence of antibiotic resistance, photodynamic therapy has become a viable alternative therapy for bio-film related diseases such as dental caries. The advantages of photodynamic therapy over conventional antimicrobial agents are first, rapid killing of target organism depending mainly on the light energy dose delivered and therefore the power output of the light source used. Hence, resistance development would be unlikely as killing is mediated by singlet oxygen and free radicals and high concentration of photosensitizer do not need to be maintained in the disease site for more than a few minutes, in contrast with hours or even days necessary in the case of conventional antimicrobial agents. Finally, antimicrobial effects can be confined to the site of the lesion by careful topical application of photosensitizer and the area of irradiation can be restricted further by using an optical fibre [73].

The results of this experimental investigation showed that LED light in the presence of TB-O would be an effective approach against dental plaque -related diseases. Another new application of this study is the use of nanoparticles in a combined therapy approach that combines radiation therapy with PDT, a technique called self-lighting

photodynamic therapy (SLPDT). Taken together, the studies discussed here suggest that this novel approach is promising and might represent a new direction in the near future. It should be noted that nanoparticle-based PDT is still in its infancy and much remains to be learned. This represents the most innovative approach that entails the up conversion of nanoparticles which are able to convert low-energy radiation to higher-energy emissions, thereby enabling PDT in deep tissues.

This study has again demonstrated a superior bactericidal effect of silver nano particles against organisms in conjunction with LED laser irradiation would be an effective protocol against pathogens like *S.mutans* and *L.acidophilus* that may resist many antimicrobial agents.

Much work still needs to be done as very few clinical studies have evaluated the effect of the different delivery systems in terms of clinical efficiency and for application purposes. Beyond the laboratory Petridish, this technology still needs answers like appropriate dosage, delivery systems and exposure times that maximize clinical effectiveness while minimizing side effects.

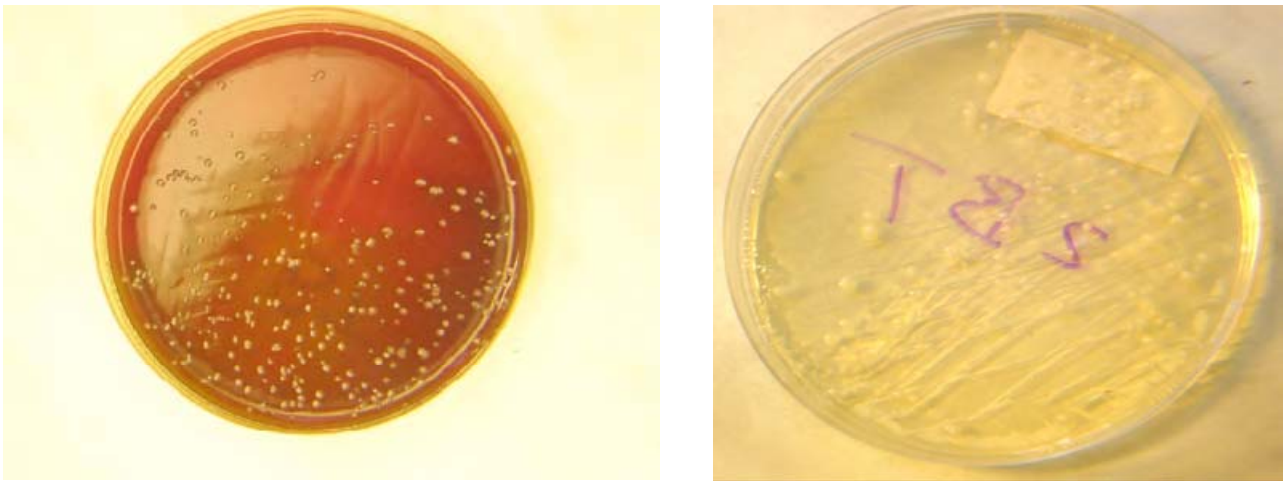
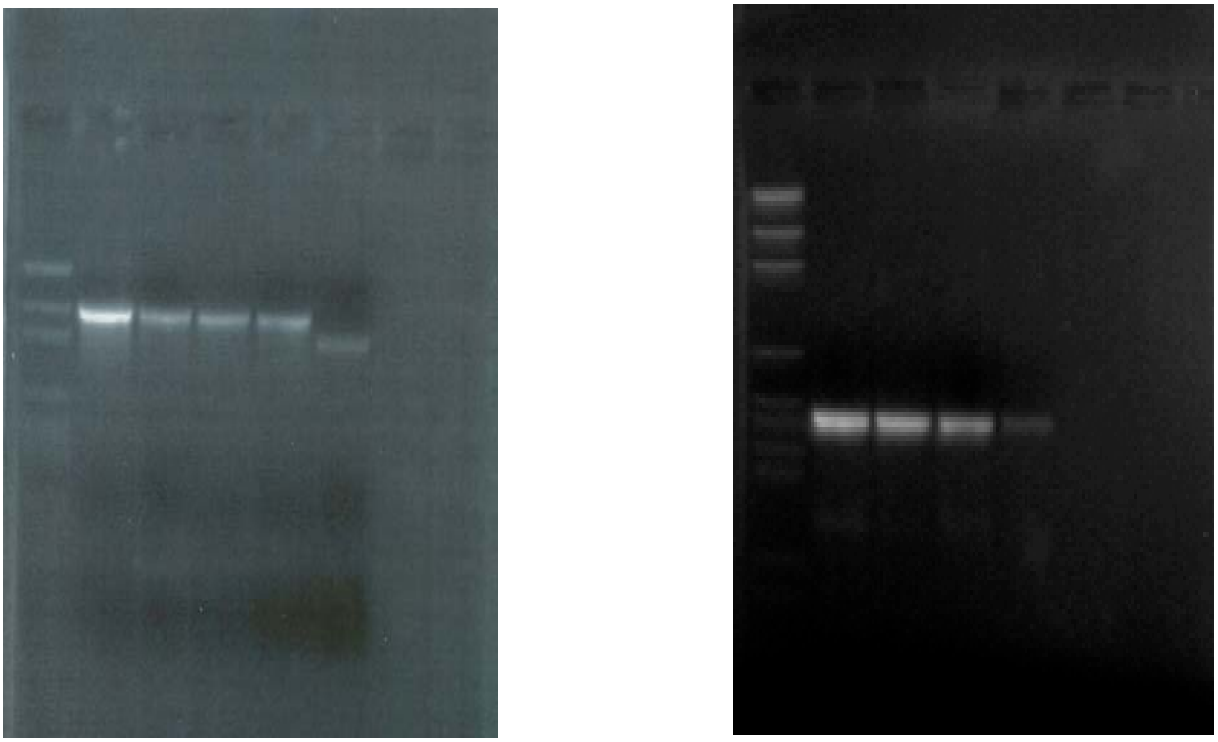


Fig4.1 Selective Isolaion of *L.acidophilus and S. mutans*



S.mutans **L.acidophilus**

Fig 4.2,PCR Identification of *S. mutans and L.acidophilus*

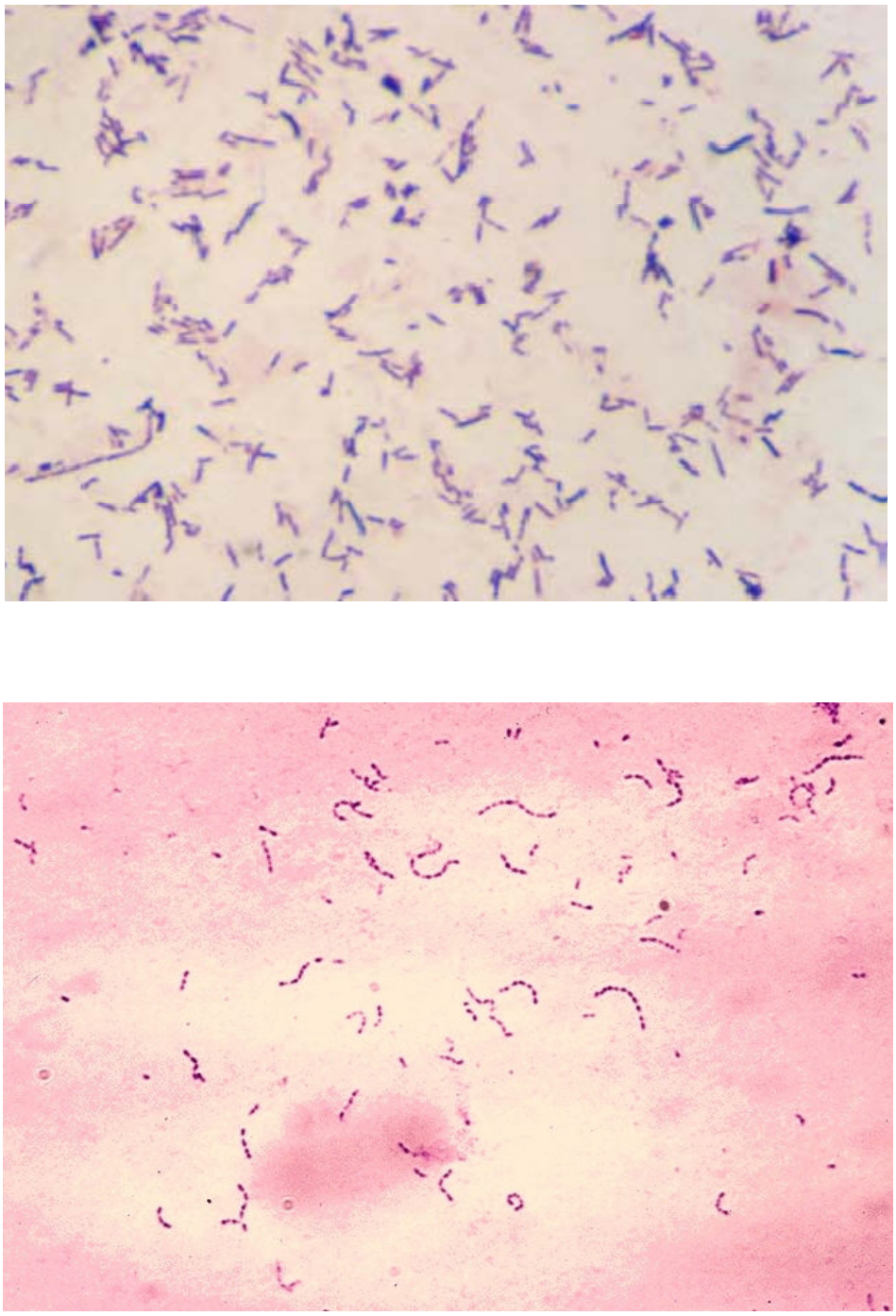


Fig 4.3 Gram staining of L.aciophilus and S,mutans

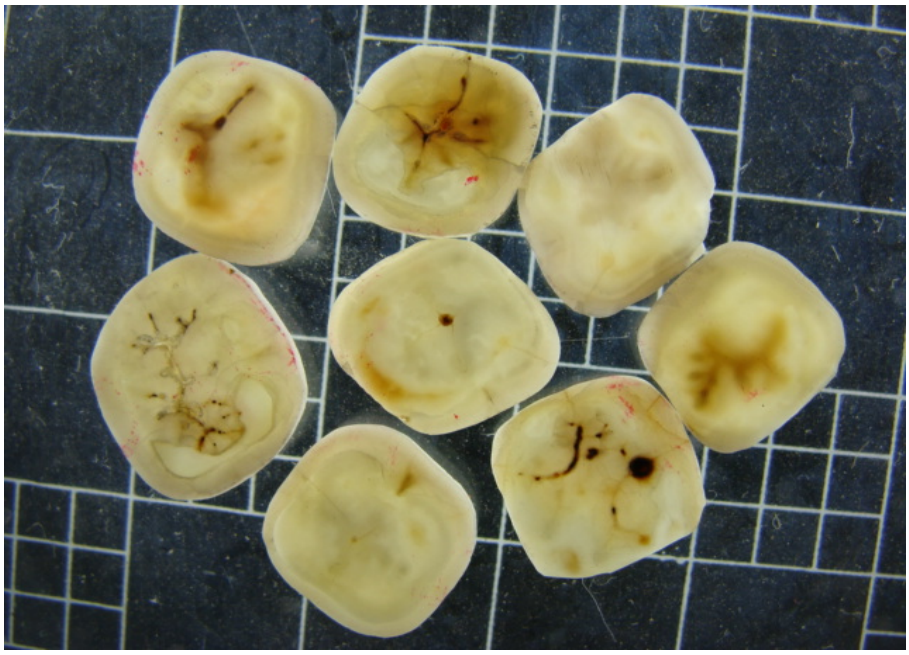


Fig 4.4 Dentin slab specimen

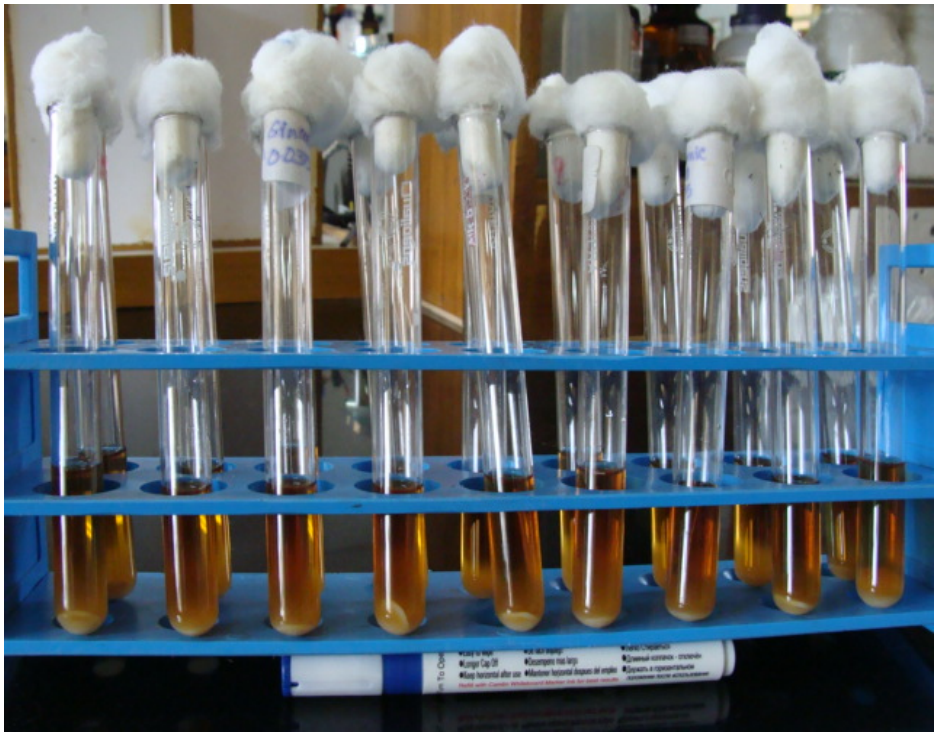
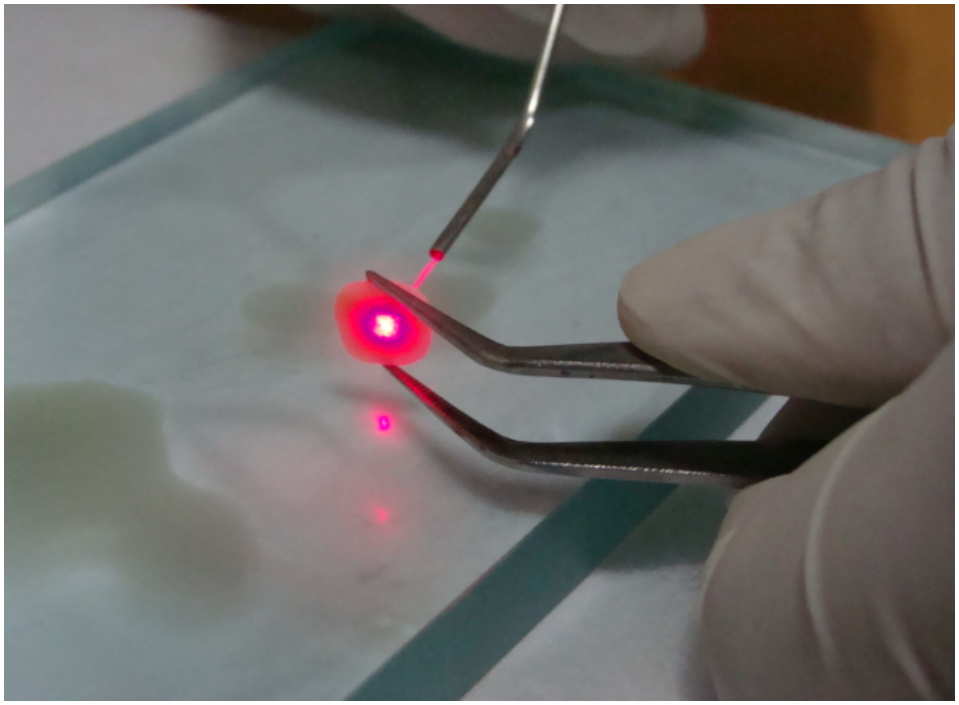


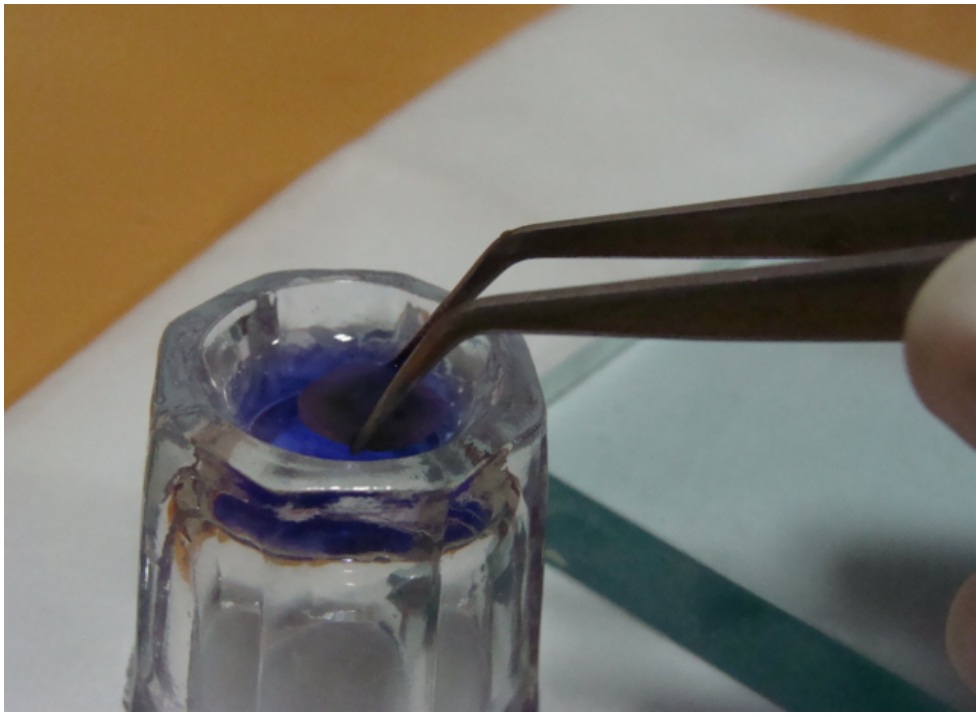
Fig4.5 Bacterial inoculation of the Dentin specimen

Fig 4.6 Test Groups

Fig 4.7 LED Laser Treatment of the Specimens



Group III-LED ONLY



TB-O treatment

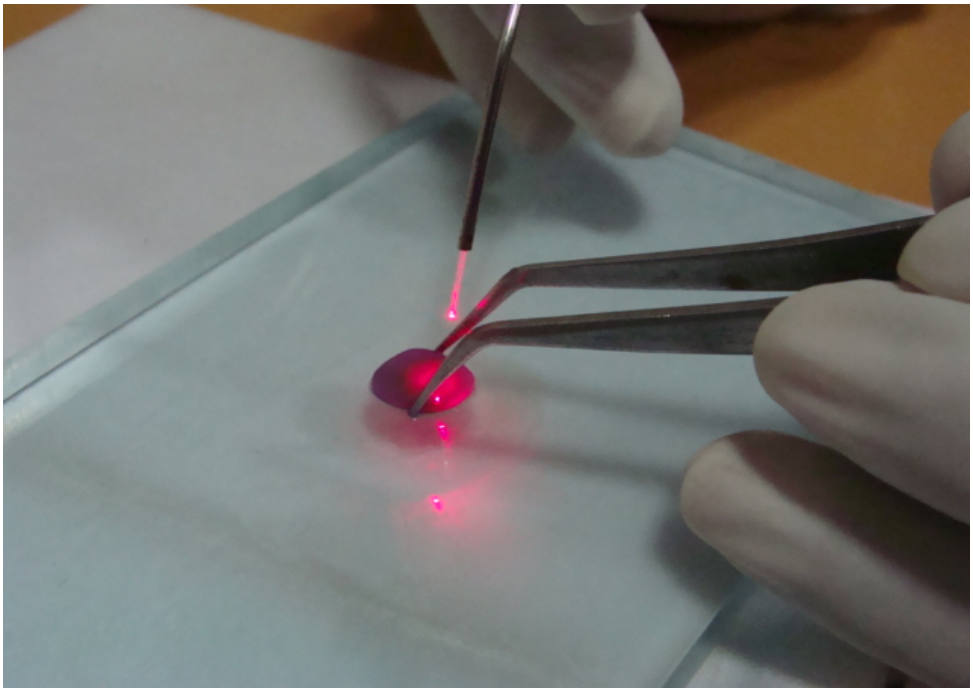
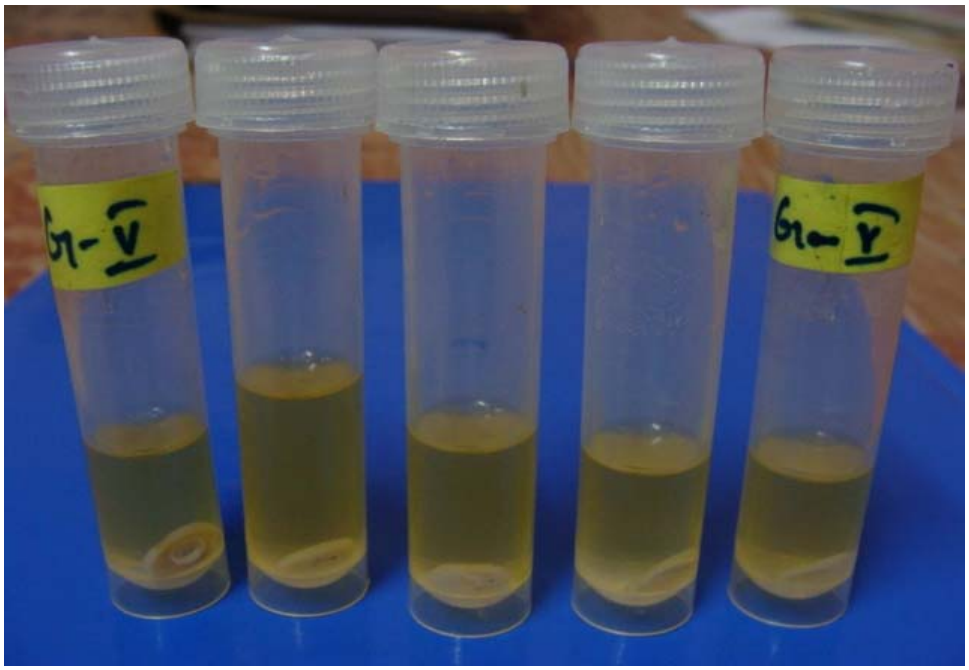


Fig-4.8 Group IV-LED+DYE



Group-V-Nanosilver solution

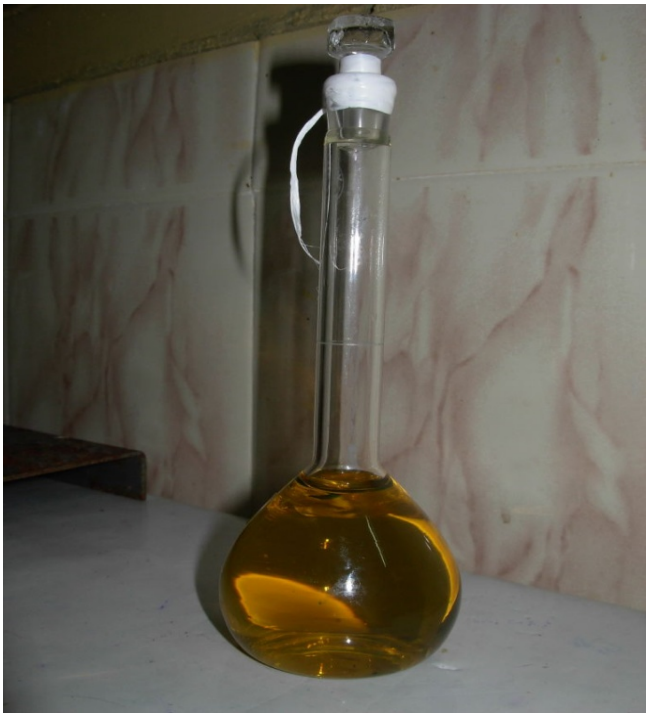


Fig-4.9 Nanosilver solution

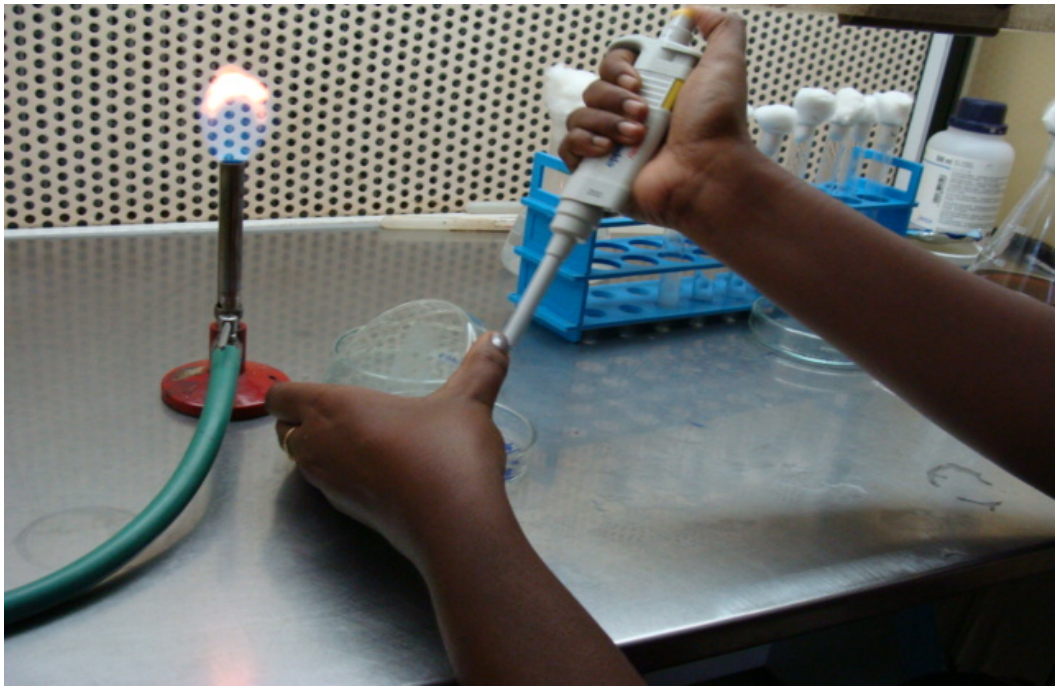
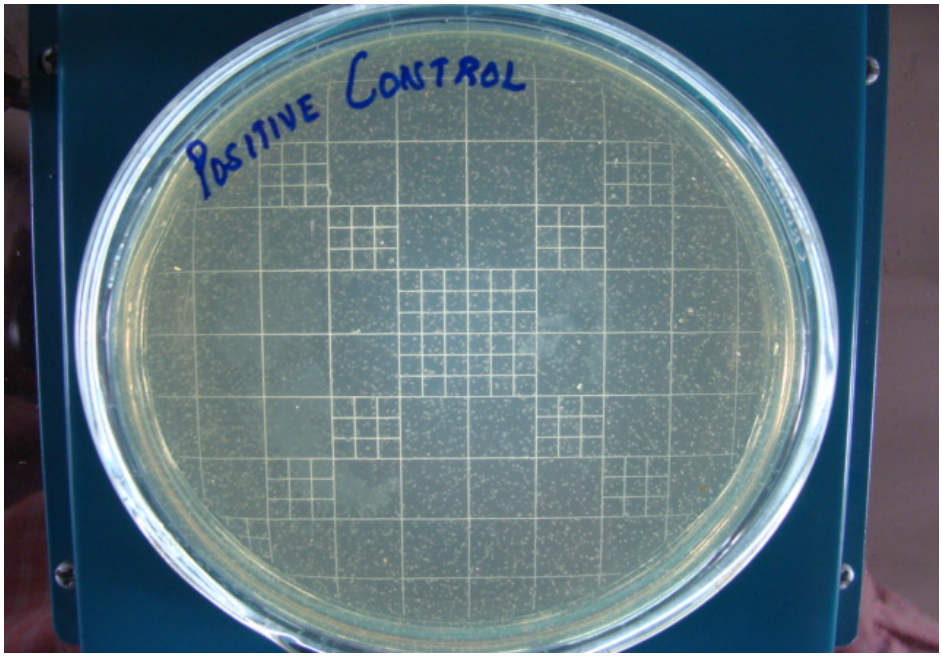


Fig 5 Bacterial Inoculation in UV chamber

Fig 5.1 CFU of the Dentin Specimen:



Group II Positive control

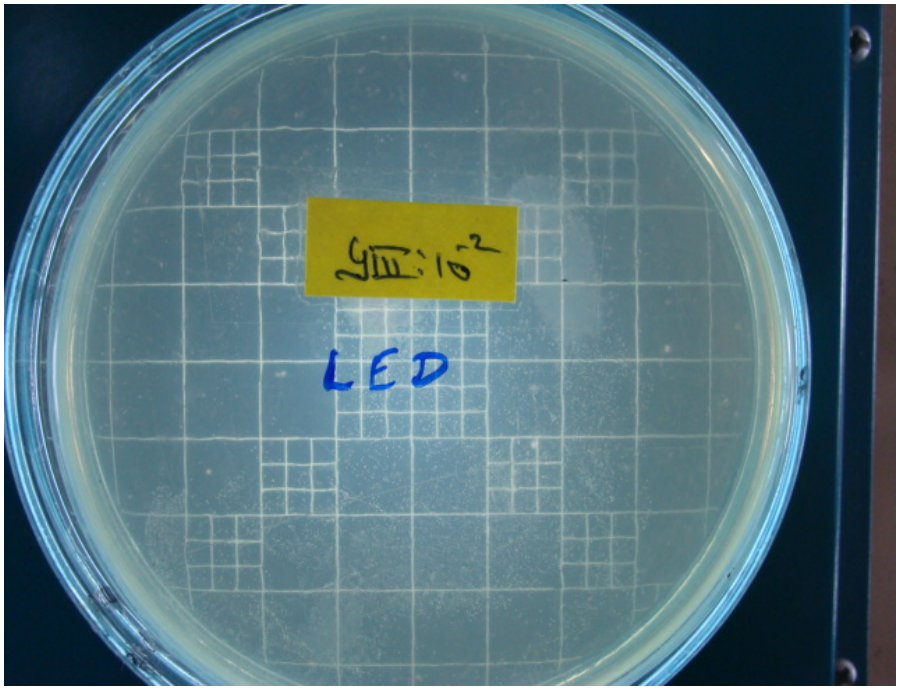


Fig-5.2 Group III-LED

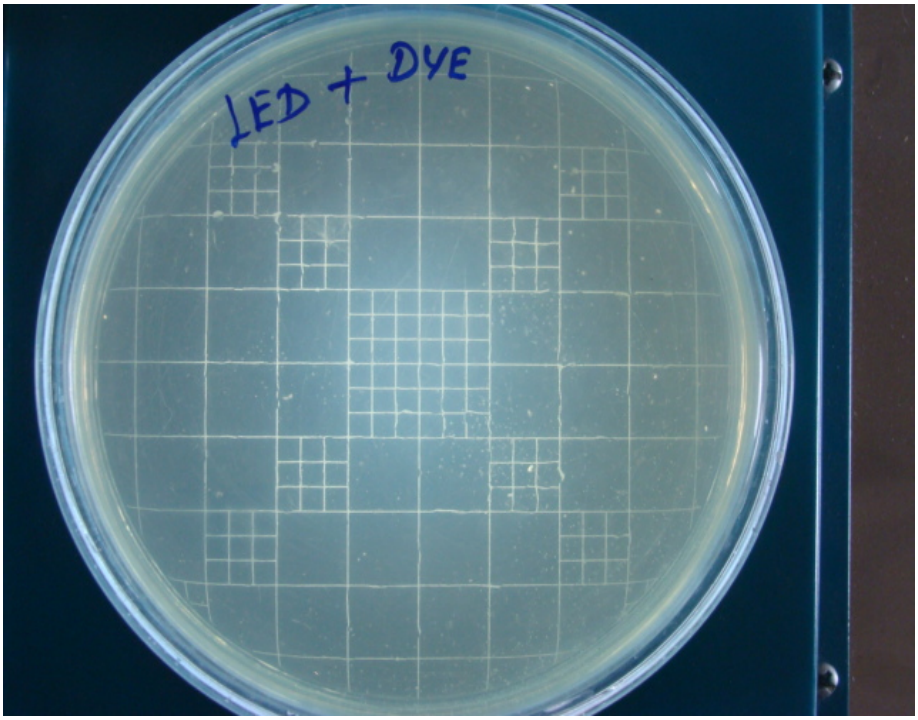
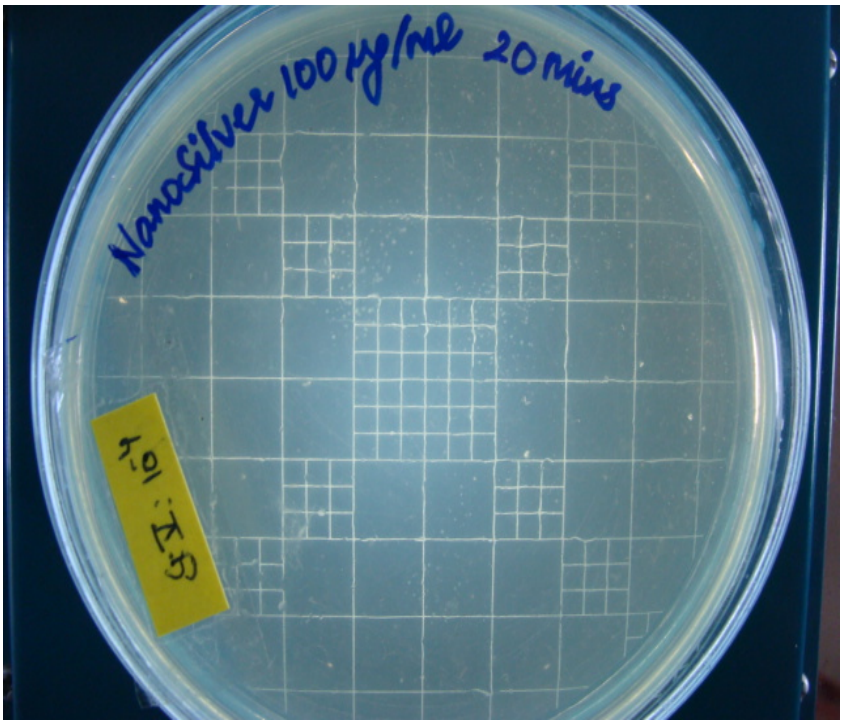
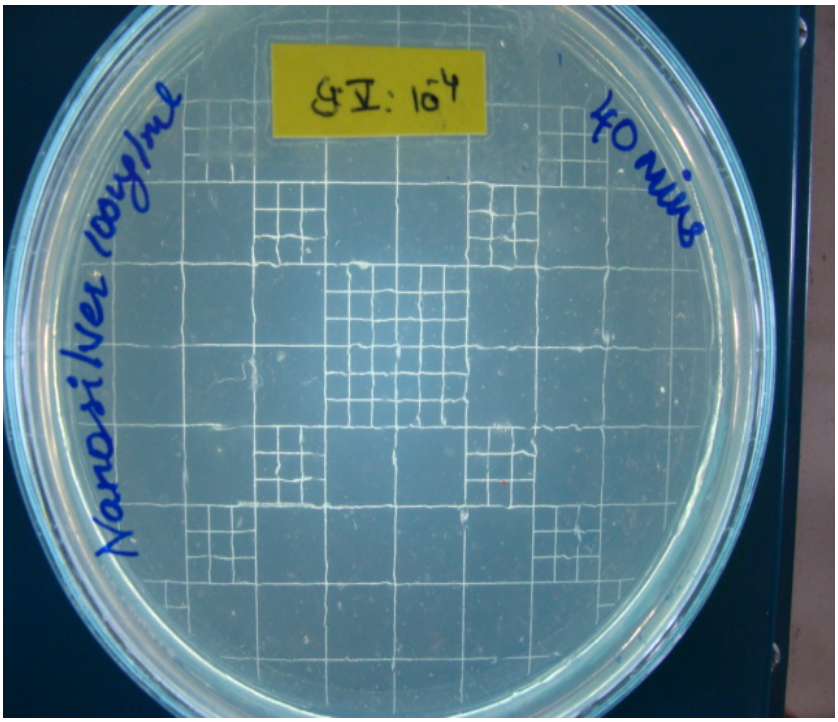


Fig-5.3 Group IV-LED+DYE

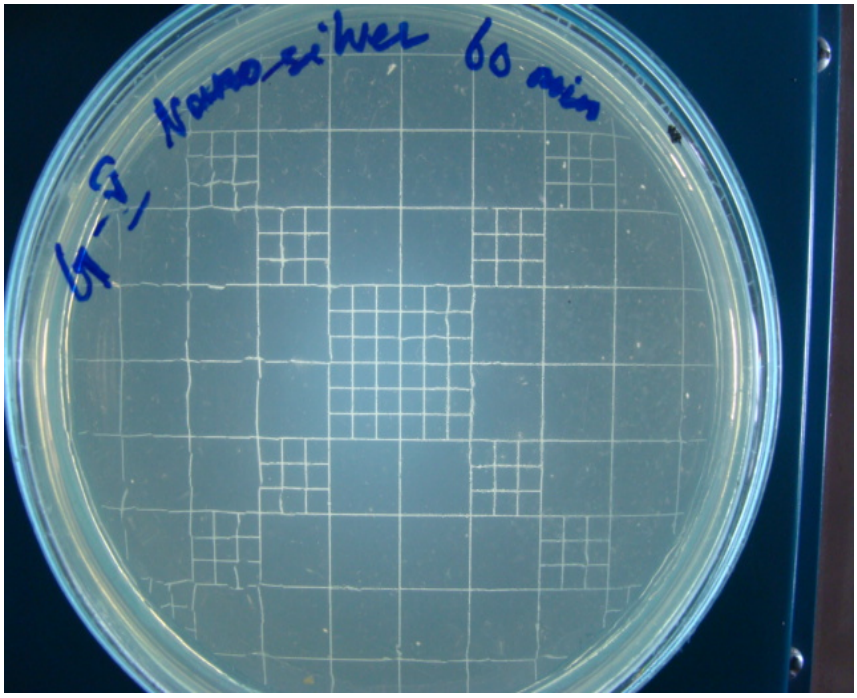
Fig-5.4 Group V: NANOSILVER



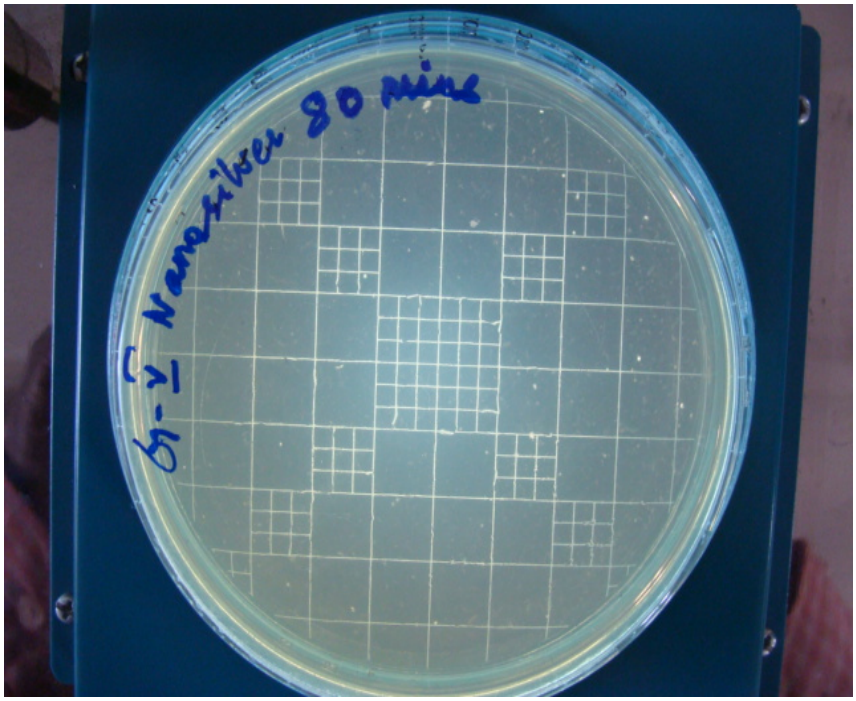
20 seconds



40 seconds



60 seconds



80 seconds

Fig-5.5 **Group VI:LED+DYE+NANOSILVER**

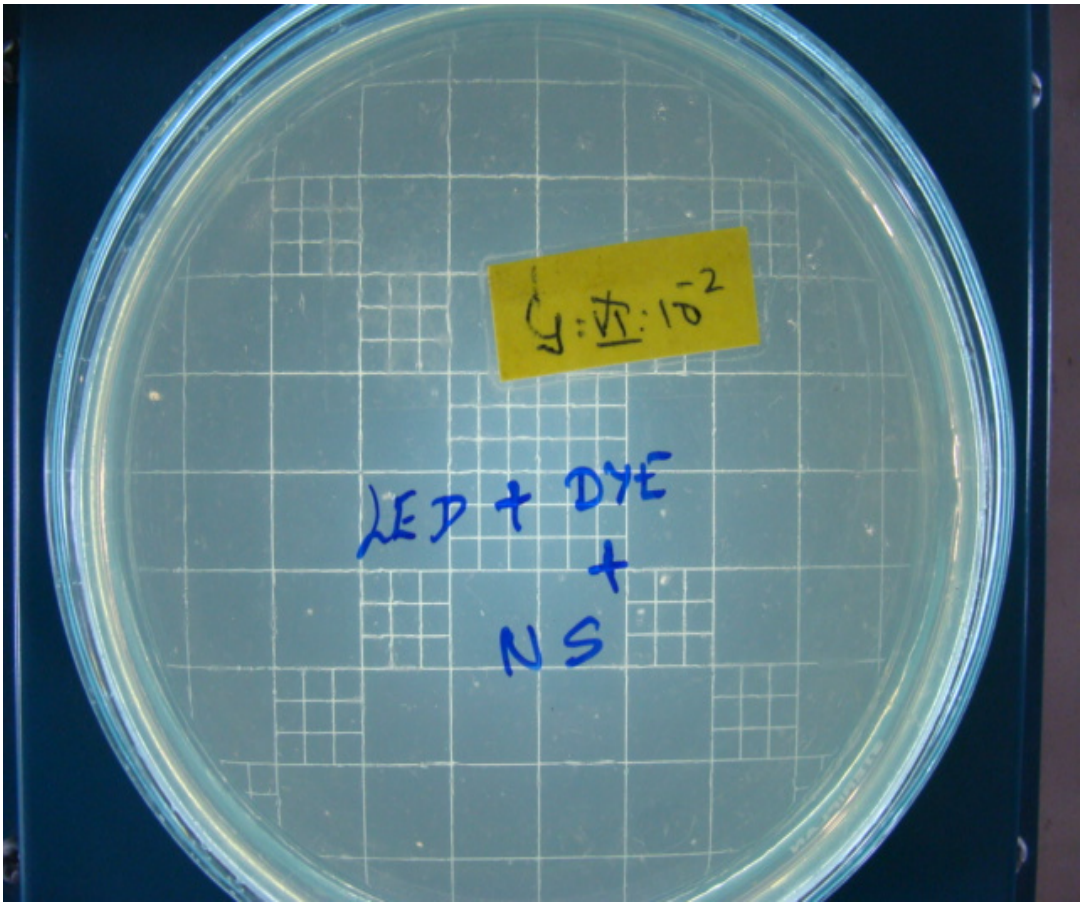
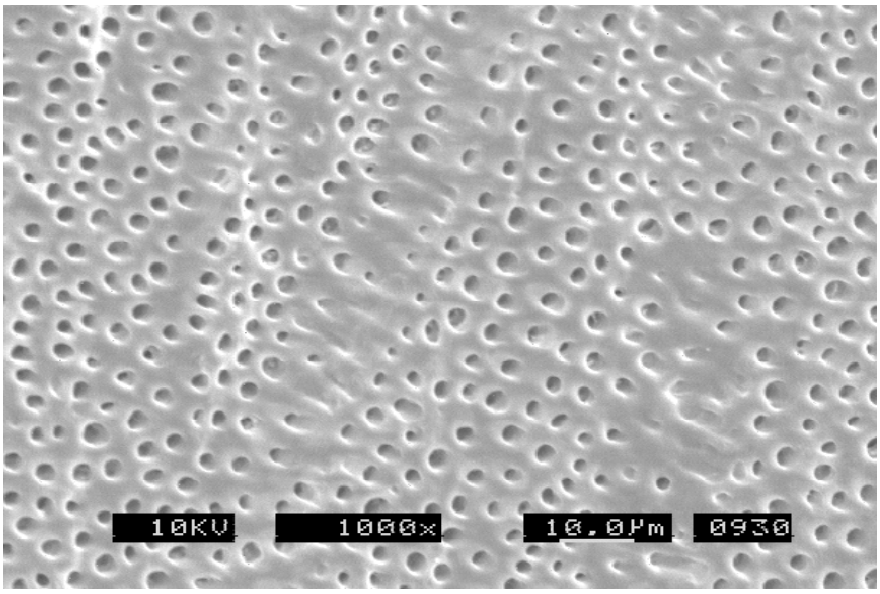
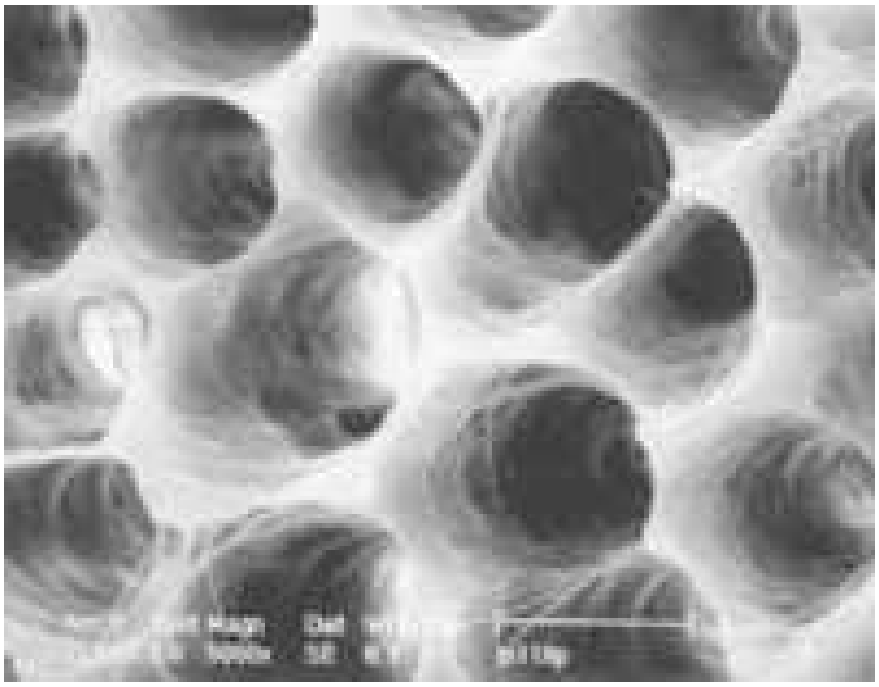


Fig 5.6 ESEM Examination:Group- I

Negative control





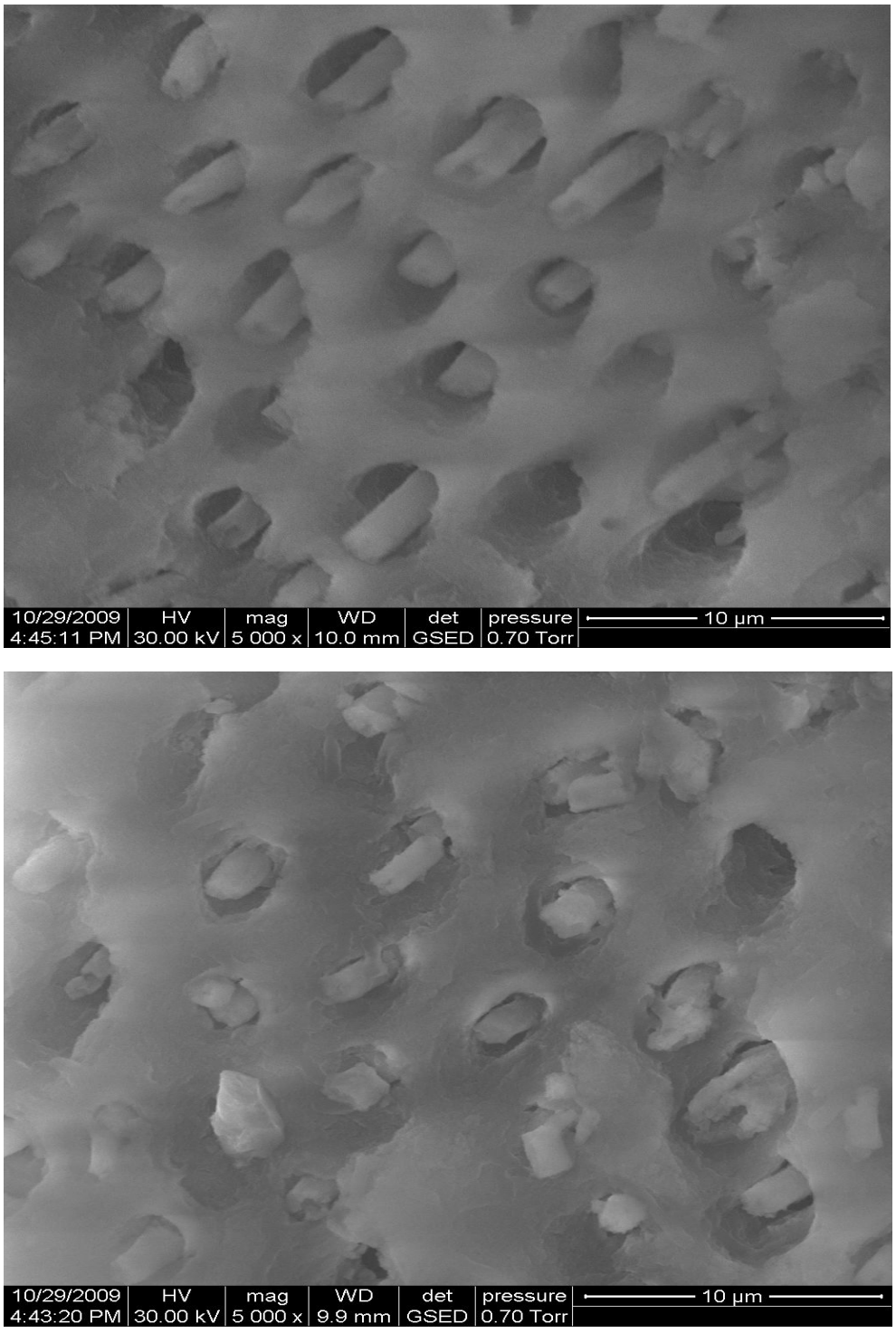
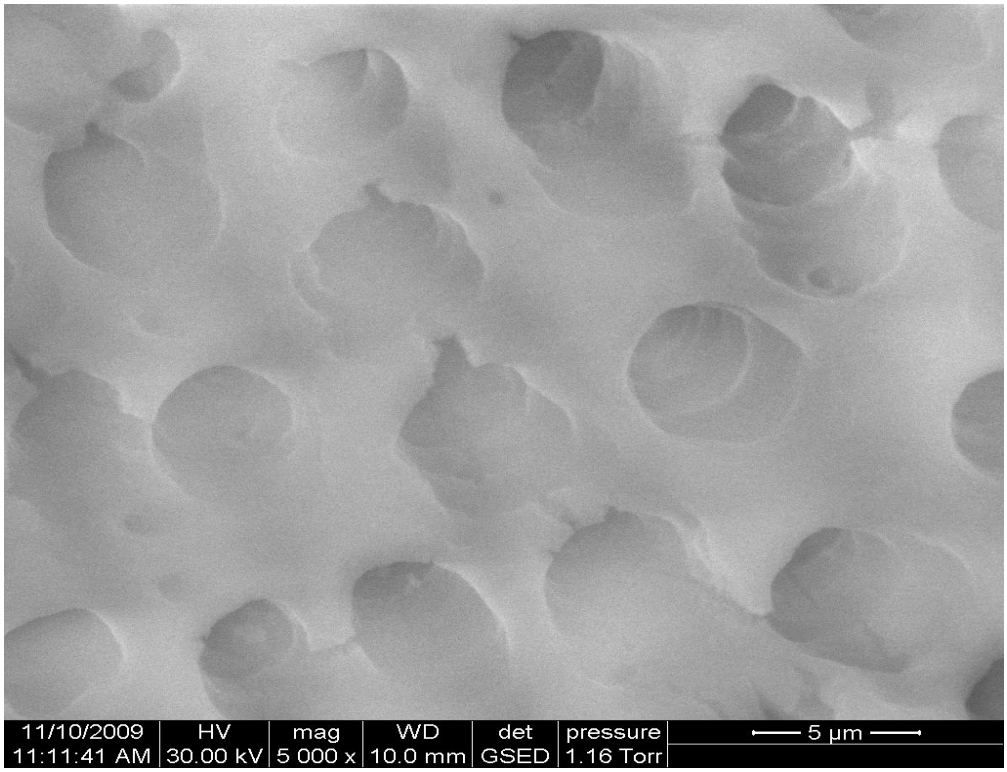


Fig-5.7 Group II- positive control



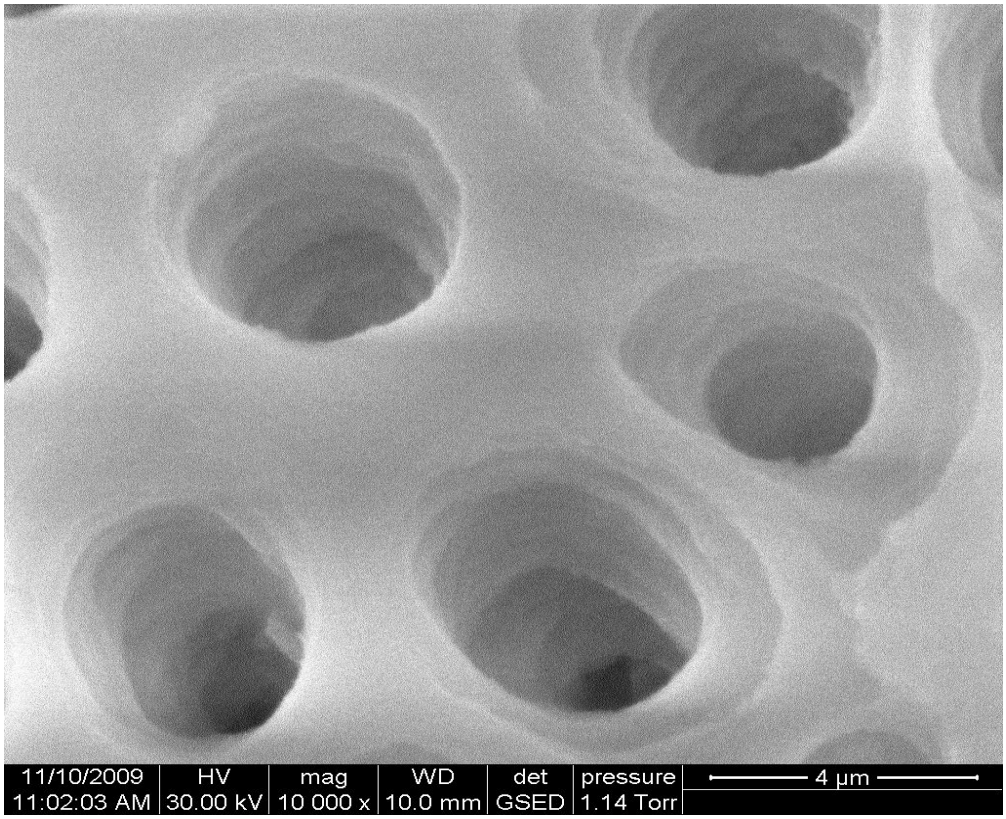
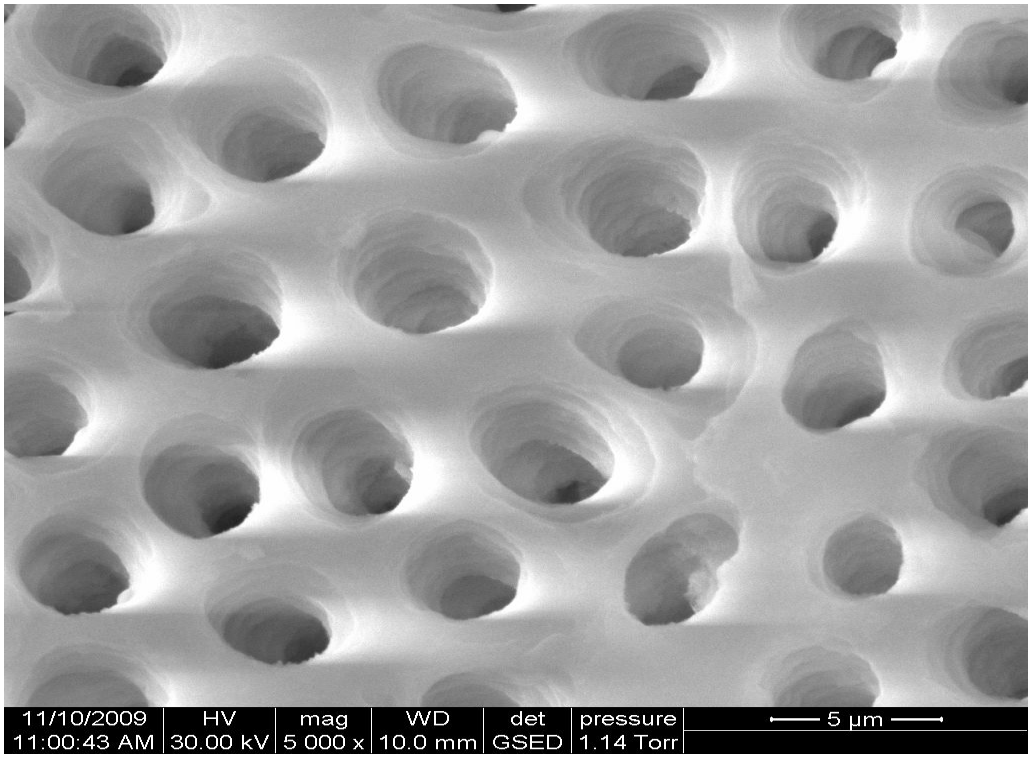


Fig-5.8 Group III-LED ONLY



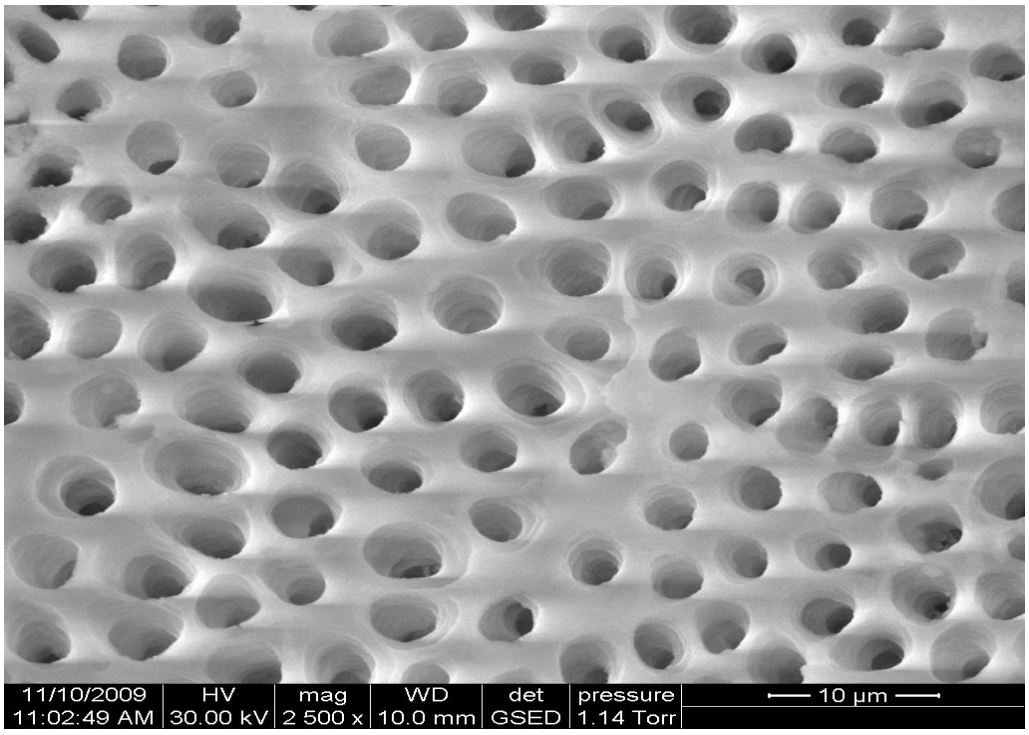
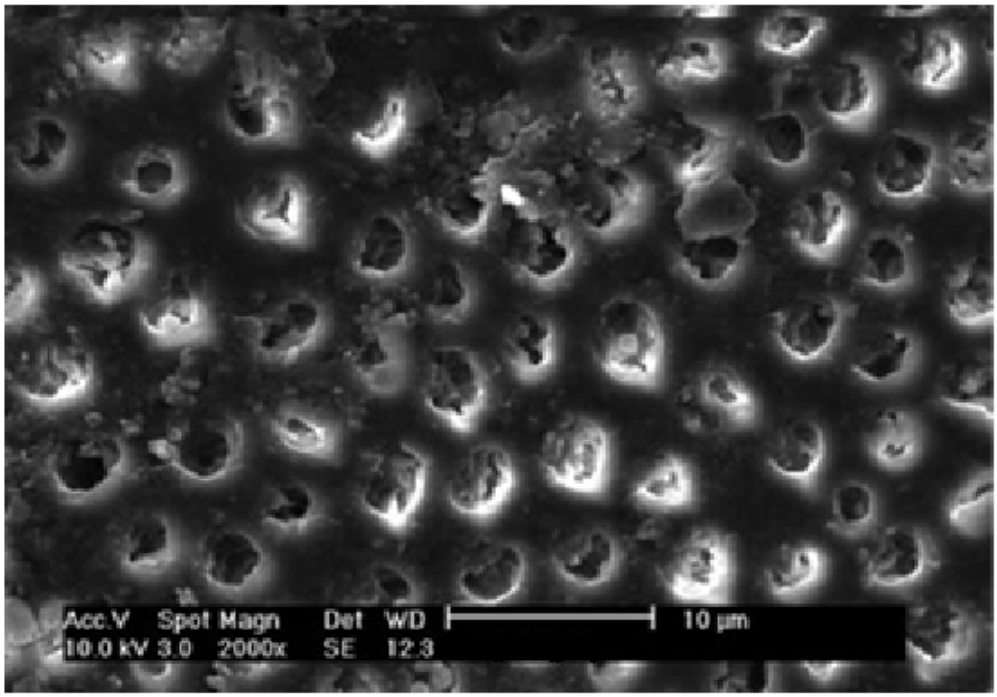


Fig-5.9 Group IV-LED+DYE



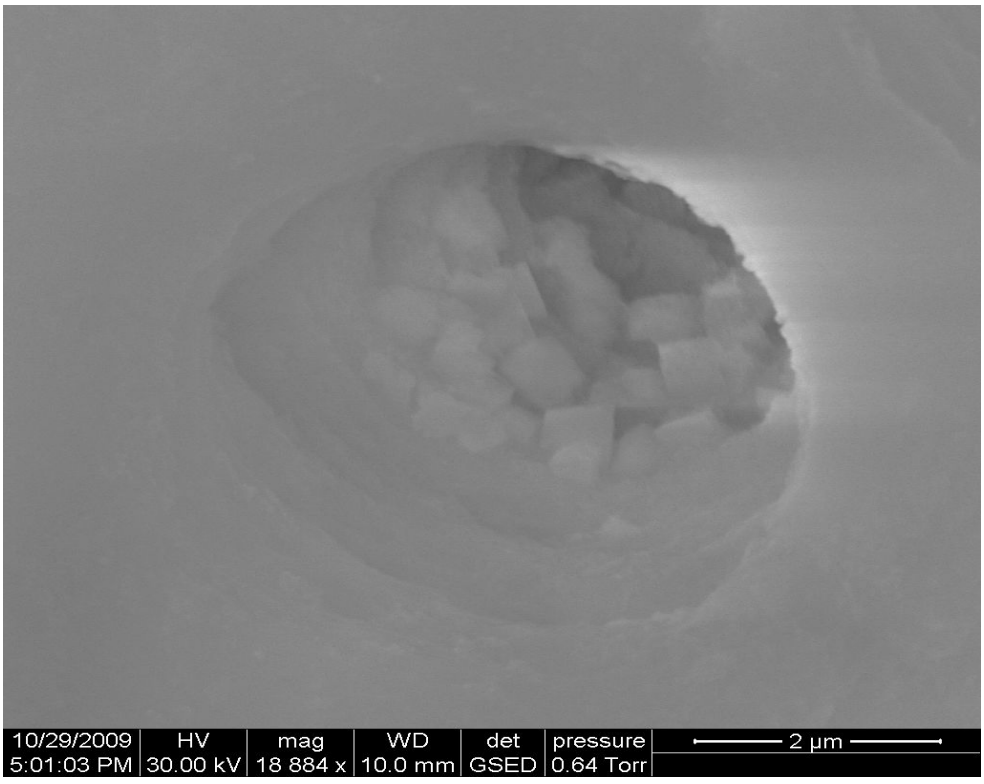
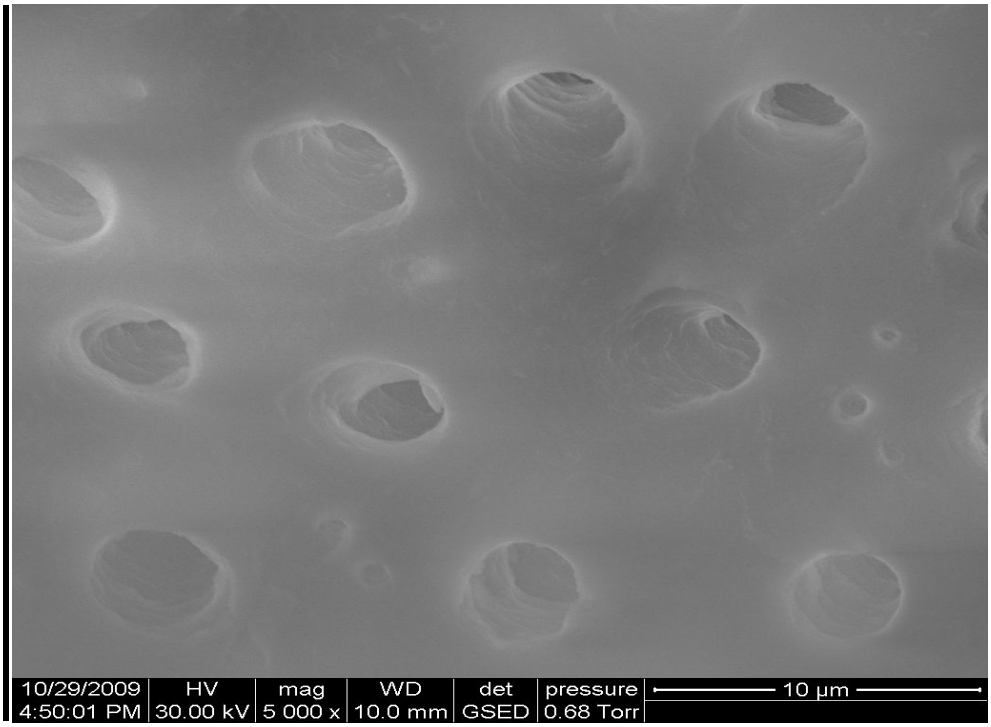


Fig-6 Group V-NANOSILVER ONLY



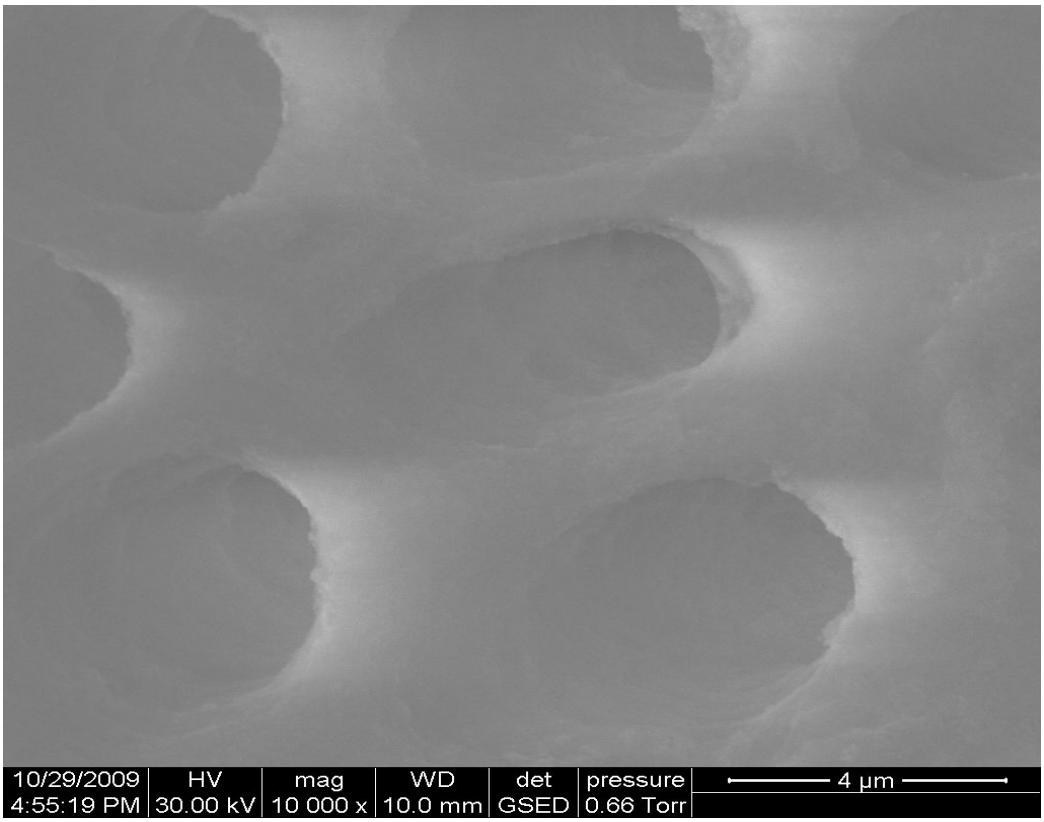


Fig-6.1 Group VI-LED+DYE+NANOSILVER

Reagents and Equipments :

Fig 3.2 Disinfection solution

Fig 3.3 TBO

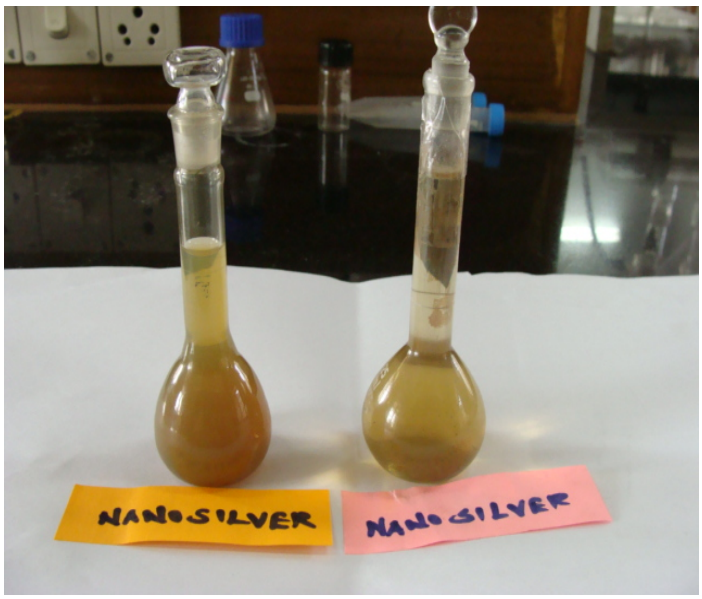


Fig 3.4 Nanosilver solution

Fig 3.5 UV spectrophotometer

Fig 3.6 Phase contrast microscope

Fig 3.7 Digital counter

Fig 3.8 LED laser

Fig 3.9 ESEM Apparatus

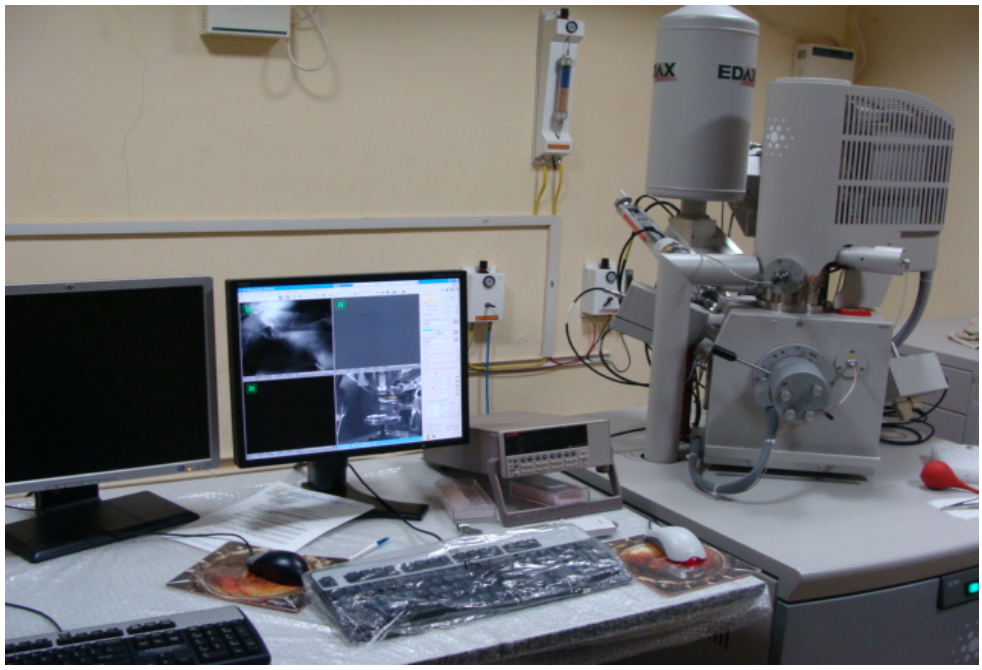


Fig 3.10 Hard tissue microtome

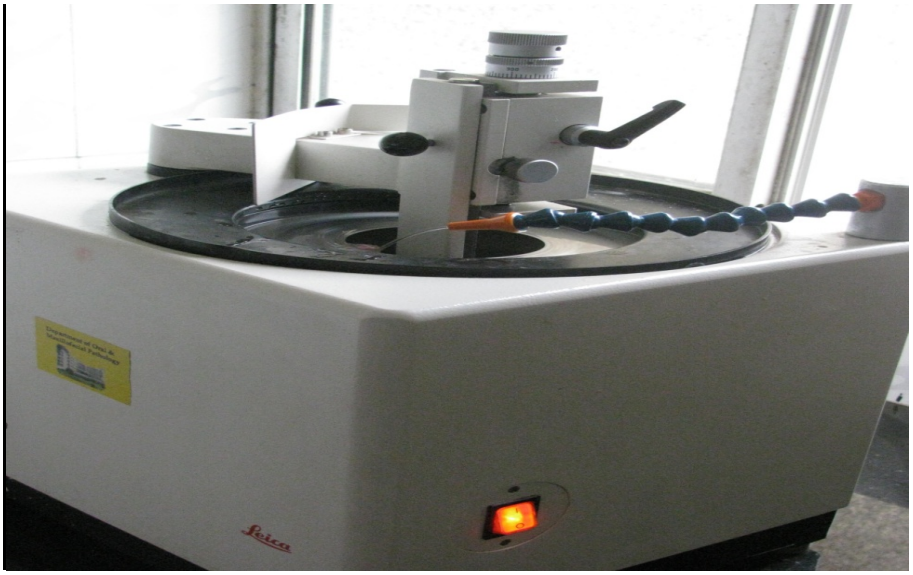


Fig-3 **IMTECH –CHANDIGARG:MTCC:FREEZE DRIED FORM OF S.MUTANS & L.ACIDOPHILUS**

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